Biochemical Studies on Entamoeba histolytica with Special Reference to its Host Invasive Functions

(SUMMARY)

A THESIS SUBMITTED
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ALIGARH MUSLIM UNIVERSITY, ALIGARH
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BY
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Entamoeba histolytica attained considerable medical interest with the discovery of Losch, a Russian physicist, in 1875, that this amoeba was the cause of dysentery in man. However, many infected individuals showing presence of cystic and tropic forms of the amoeba in their stools, were often found to be free from any symptoms of this disease. Variable invasive potency of different **E.histolytica** isolates has also been seen in experimental animals and quite often such cultures, which were initially pathogenic, lose their virulence on prolonged cultivation particularly under axenic conditions as tested in conventional animal models. Such attenuated cultures may, however, generate pathological manifestations in specially devised models and generally regain invasiveness by repeated cultivation in cholesterol enriched culture media.

The main interest of the present study was to investigate biochemical differences in 'invasive' and 'non-invasive' **E.histolytica** strains and modification of such parameters by cultivation of amoeba in cholesterol enriched medium. Further, salient properties of these biochemical/physiological functions including their sensitivity to specific activators/inhibitors and antiamoebic drugs were also investigated.

STRAIN VIRULENCE

The **E.histolytica** cultures used in the study viz., DKB, NIH:200 and IP:106 were all obtained originally from acute amoebiasis patients

but DKB and NIH:200 showed a much lower degree of virulence compared to IP:106 as tested in terms of lesion forming ability in the liver of hamsters. Further, the capacity of NIH:200 strain to infect animals (hamsters) was found to increase considerably after its subculturing in cholesterol enriched medium. The same order of virulence was indicated from tests of Concanavalin A (Con A) agglutinability of the amoebic cells and haemolytic activity of their homogenates, which are in vitro indices of **E.histolytica** virulence.

ERYTHROPHAGOCYTOSIS

The virulent IP:106 culture showed significantly higher erythrophagocytotic capacity as compared to DKB and NIH:200 strains. Statistically significant differences were, however, indicated in the clonal cultures derived from the DKB strain which exhibited lowest erythrophagocytotic capacity amongst the three strains. The same DKB clone (DKB-3) which showed maximum erythrophagocytotic capacity was found earlier in our laboratory to possess highest haemolytic capacity, Con A agglutinability and acid hydrolase activities amongst the tested clones. Serial passage of NIH:200 culture through cholesterol-enriched medium resulted in considerable increase in the erythrophagocytotic capacity of the culture. The same relative order of phagocytosis was indicated in the three cultures (DKB, NIH:200 and IP:106) when ^{14}C-sucrose-loaded RBC ghosts were supplied to amoebic cells and uptake of radioactivity was measured.

A number of sugars viz., galactose, N-acetylgalactosamine, lactose and fructose inhibited erythrophagocytosis in both NIH:200

and IP:106 strains of **E.histolytica**. N-acetylgalactosamine also reduced phagocytosis of ^{14}C-sucrose loaded RBC ghosts.

The rate of $^{14}CO_2$ production from ^{14}C-U-glucose was considerably higher in IP:106 as compared to NIH:200 and increased when the latter was cultivated in cholesterol enriched medium. Addition of RBC stimulated this activity of **E.histolytica** in all the three cultures (IP:106, normal and cholesterol passaged NIH:200); maximum increase being shown by IP:106. Further, a similar pattern of relative ^{125}I-labelled Con A internalization was noted in these cultures which showed time dependence and marked reduction in presence of α-methyl mannoside (specific hapten of Con A), while bovine serum albumin on the other hand elicited stimulation.

OXIDO-REDUCTIVE FUNCTIONS

E.histolytica can reduce nitroblue tetrazolium (NBT) in Hank's balanced salt solution to almost the same extent as in Eagle's medium. Further, this was stimulated only to a minor degree by substrates viz., glucose, pyruvate and DL-serine, known to support its respiratory activity (O_2 uptake). However, both NADH and NADPH increased NBT reduction several fold, the effect being greater with latter. A sizable proportion to this endogenous dye-reducing capability was associated with low speed sediments obtainable from amoebic homogenates which shared also the bulk of ^{125}I label (when the homogenate were prepared after surface-labelling of the cells with Na ^{125}I). Conversion of the dye to formazan was strongly inhibited by -SH blocking agents but was not influenced by rotenone and antimycin A. The activity

was inhibited also by H_2O_2 while catalase stimulated this. Superoxide dismutase only slightly curtailed NBT reduction in intact cells, but inhibited this in homogenates in a concentration dependent manner to a maximal extent of 35%. Almost the same degree of curtailment in this activity was induced by anaerobic conditions. Both phorbol myristate acetate and concanavalin A stimulated the activity in intact cells; the effect of the latter being nullifiable by α-methyl mannoside.

This substrate-independent reduction of NBT was strongly inhibited by antiamoebic drugs, phanquinone and quinacrine, and also by metal chelator, o-phenanthroline.

Mixture of amoebic and red blood cells reduced NBT to a much higher extent than total reduction accounted in terms of the activities observed with these two types of cells separately indicating thereby that erythrophagocytosis stimulates reduction of NBT in amoeba. Further, addition of Escherichia coli to the **E.histolytica** cell suspension also increased the rate of O_2 uptake by this amoeba.

Comparison of specific activities of NBT reduction and alcohol dehydrogenase in the virulent IP:106, the normal and cholesterol passaged NIH:200 showed more than two fold higher values of both these parameters in IP:106 and cholesterol passaged NIH:200 as compared to normal (unpassaged) NIH:200. The alcohol dehydrogenase activity highly favoured production of alcohol from acetaldehyde and the backward reaction showed a very poor rate. Further, the enzyme showed a much higher activity in presence of NADH as compared to NADPH. This activity of **E.histolytica** was highly sensitive to -SH blocking agents.

It has been postulated, on the basis of above results, reactive oxygen metabolites generated by **E.histolytica** are potentially self-injurious. However, the parasite can readily detoxify them at the cost of suitable adherable/phagocytosable host or bacterial cells and can thus use them as 'weapons' in the host system.

ENZYMES RELATED TO E.HISTOLYTICA VIRULENCE

Greater acid phosphatase activity in the homogenates of IP:106 as compared to NIH:200 and its stimulation in the latter through cultivation in cholesterol enriched medium was reported earlier from our laboratory. This finding was confirmed in the present investigation.

A comparison of total acid phosphatase content of **E.histolytica** cells (as assayed in its homogenates) versus the enzyme activity released in growth medium at different post-inoculation intervals indicated progressive increase with time both in cell number and total enzyme content of harvested amoebic populations as well as enzyme activity released in the medium. The enzyme content per cell did not show much variation at different culture ages, however, total extracellular levels of the enzyme were several fold higher as compared to the activity of the cellular populations at all the post-inoculation intervals suggesting that the enzyme is actively secreted by these cells in the growth medium. Triton X-100 did not influence the enzyme activity in either cellular homogenates or cell free medium.

Fractionation of the precipitate obtained from the cell-free medium at 60% ammonium sulfate saturation by DEAE-cellulose chromatography yielded 3 distinct peaks (AP_1, AP_2 and AP_3) of the enzyme

activity on stepwise elution with 0.05, 0.1 and 0.2 M sodium chloride. The PAGE of the enzyme in AP_1 gave 1 enzyme band (Rm: 0.46), 2 bands in AP_2 (Rm : 0.46 and 0.61) and 3 bands in AP_3 (Rm: 0.46, 0.64, 0.66).

The activities of three peaks showed resistance to tartarate, but was inhibited by fluoride, cupric chloride, ethylenediamine tetraacetic acid, ammonium molybdate and cysteine. Concanavalin A however, caused a concenntration dependent inhibition of enzyme in AP_1 but stimulated this activity in AP_2 and AP_3. Glucose (2 mg/ml) reversed this stimulatory or inhibitory effect of Con A. All tested antiamoebic drugs were inhibitory towards this enzyme of **E.histolytica**.

A pattern similar to that of acid phosphatase was noted for relative specific activities of proteinase in IP:106 and normal and cholesterol passaged NIH:200 using azocasein as substrate. This was predominantly associated with the sedimentable fraction (10,000xg, 20 min) and was significantly stimulated by 0.5% triton X-100. Further, it showed optimum activity at pH 6.0 and was highly sensitive to -SH blocking agents, Zn^{+2} and Cu^{+2} while β-mercaptoethanol and cysteine acted as stimulants. Glutathione (red.), L-cysteine and β-mercapto-ethanol reversed the inhibitory action of p-chloromercuribenzoate and N-ethylmaleimide. Antiamoebic compound, phanquinone, also inhibited this activity.

PYRUVATE PHOSPHATE DIKINASE

Generation of pyruvate from phosphoenolpyruvate in **E.histolytica** (NIH:200) is carried out by pyruvate phosphate dikinase (PPD), an

enzyme which is characterized by requirement of pyrophosphate and AMP instead of ADP in case of functionally analogous mammalian pyruvate kinase. The enzyme of **E.histolytica** showed optimal activity at pH 6.7 and was highly sensitive to p-chloromercuribenzoate and N-ethylmaleimide but not iodoacetate. Both EDTA and EGTA significantly stimulated PPD activity and Ca^{+2} and Zn^{+2} were inhibitory. Antiamoebic compound phanquinone was highly inhibitory (70% inhibition at 10 μM concentration) while diodoquin also caused slight inhibition (40% at 60 μM concentration). The inhibition due to phanquinone was non-competitive in nature (Ki: 2.91 μM). In contrast, the pyruvate kinase activity of rat liver homogenate was only slightly inhibited (10%) at 5 μM phanquinone concentration and the effect showed little further increase even at a drug concentration as high as 30 μM.

The study has generally inidcated that the virulence of **E.histolytica** may not be linked to any single biochemical function but may depend on a multitude of factors which are all stimulated through cultivation of avirulent strains in cholesterol enriched medium, in a bunched manner. This lends support to the view that virulent sub-populations may in fact preexist in such cultures which get enriched through some selective process in presence of high level of cholesterol in the growth environment. The work also suggests that thiol groups may be involved in several important functions of this amoeba. This feature and specific requirement of pyrophosphate by this parasite may be useful in developing new chemotherapeutic strategies against this pathogen.

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C E R T I F I C A T E

This is to certify that the work in this thesis entitled "**Biochemical Studies on Entamoeba histolytica with Special Reference to its Host Invasive Functions**" has been carried out by MR. SHAILENDRA KUMAR, M.Sc.,M.Phil.(Biochem.) under our joint supervision.

He has fulfilled the requirements of the Aligarh Muslim University regarding the prescribed period of investigational work for the award of Ph.D. degree.

The work included in this thesis is original unless stated otherwise, and has not been submitted for any other degree.

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C O N T E N T S

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PREFACE

The world of parasites present a unique and diverse panorama of biological species. The interactions of a variety of parasites with man are major human health and hygiene problems all over the world, especially in tropical countries like India, where a wide variety of parasitic infections like amoebiasis, filariasis, malaria, leishmaniasis and leprosy pose major health hazards.

Amoebiasis, which is caused due to **Entamoeba histolytica**, is a major health problem in the Indian sub-continent, the South-East Asia, China, Mexico, Eastern part of South America and West and South-East Africa. The disease is more closely related to sanitation and socioeconomic status than to climate.

The primary site of **E.histolytica** infection in man is the intestine. A common clinical manifestation in amoebic dysentery is diarrhoea with/without bloody mucus. Other forms of intestinal amoebiasis are fulminating amoebic colitis, amoeboma of the colon and amoebic appendicitis. The amoebae may also migrate from intestine to infect other tissues, the most prevalent form of extra-intestinal amoebiasis being amoebic liver abscess.

The parasite (**E.histolytica**) has an indistinguishable dual character pertaining to its virulent and avirulent forms. As a virulent form its importance lies in its intestinal tissue necrotic activity resulting in high incidence of dysentery and liver abscess. As an avirulent form, although its trophozoites dwell in the intestinal lumen, yet they do not invade the colonic mucosa and hence present no apparent clinical symptoms of the disease. The problem of relpse

and recurrence of infection of **E.histolytica**, despite best of the available chemotherapeutic treatments, is still a serious one.

Very limited information concerning factors affecting host-parasite relations is available. One does not know the chemical weapons employed by the parasite in invading the host. Equally interesting is the question how this parasite protects itself from the immunological mechanisms and relatively aerobic conditions while in the host. The research embodied in the present dissertation represents a humble effort to obtain some insight into such biochemical aspects of **E.histolytica** which may have special relevance to its host-invasive functions.

ABBREVIATIONS

BSA	: Bovine serum albumin
CHO	: Chinese hamster ovary
Con A	: Concanavalin A
DTT	: Dithiothreitol
EDTA	: Ethylenediaminetetraacetate
EGTA	: Ethyleneglycoltetraacetate
E-QO_2	: Endogenous oxygen uptake
Gal NAc	: N-acetyl galactosamine
MAI	: Microscopic aggregation indices
NAD	: Nicotinamide adenine dinucleotide
NADH	: Reduced NAD
NADP	: Nicotinamide adenine dinucleotide phosphate
NADPH	: Reduced NADP
NBT	: Nitroblue tetrazolium
NEM	: N-ethyl maleimide
PBS	: Phosphate buffered saline
pCMB	: para-chloromercuribenzoate
PMA	: Phorbol myristate acetate
PPi	: Inorganic pyrophosphate
Rm	: Relative mobility
SDS	: Sodium dodecyl sulphate
SOD	: Superoxide dismutase

CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

Amoebiasis, an invasive enteric illness that can spread to multiple organ systems, is caused by the protozoan **Entamoeba histolytica** (Schaudinn, 1903), which belongs to class Rhizopoda. This amoeba is carried by approximately 10% of the world's population, although only a minority of the carriers of the organism presents active clinical symptoms. The disease has a cosmopolitan distribution with greater prevalence in tropical and sub-tropical countries and remains one of the major unconquered affliction, despite the development of a variety of antiamoebic drugs.

This disease was first described in 1875 by Losch (Losch, 1875), who found a positive correlation between the number of active amoebae in the stool of a patient and the severity of the disease symptoms during the course of his illness.

Entamoeba exists in two different forms, the vegetative trophozoite and the infective cyst. The life cycle of the parasite in man (Dobell, 1928) starts with oral ingestion of the quadrinucleate cyst. These pass through the stomach and intestinal tract to reach the ileum where they excyst to form eight uninucleate trophozoites. The excysted trophozoites then continue to multiply by binary fission and it is this form that may give rise to symptoms of amoebiasis.

PATHOGENESIS/VIRULENCE OF E.HISTOLYTICA

Virulence of a given isolate depends on the intrinsic properties of the trophozoites, the growth conditions and the test system used to evaluate its pathogenicity *in vivo* or *in vitro*. The term pathogenicity is used to denote the general ability of an organism to produce disease, whereas the virulence also refers to that ability, but has a more specified connotation that implies degree (Gladstone, 1970).

Although pathogenicity of **E.histolytica** was discovered more than a century ago, the factors responsible for its virulence are yet to be properly elucidated. Clinicians have observed that human infections with **E.histolytica** are associated with diarrhoea or dysentery or may be without symptoms. The work on the virulence of **E.histolytica** showed that strains isolated from asymptomatic cases vary in their virulence from completely non-invasive to highly invasive ones (Singh *et al.*,1963; Sarkisyan and Vaskanyan,1966; Kasprazak *et al.*,1973). According to the work of Beaver *et al.*(1956) the virulence of **E.histolytica** has been determined in animals by the degree of ulceration.

Neal (1954, 1965) found that encystation does not increase virulence. He also observed that when amoebae were maintained in *in vitro* cultures for prolonged periods their virulence was lost inspite of periodic encystment.

The study of amoebic virulence has necessitated the development of suitable animal models for studying the disease. Many model systems have been used, including intestinal infection of kittens (Hoare, 1925), dogs (Faust, 1932), guinea pigs (Jervis and Takeuchi, 1979), mice and rats (Gold and Kagan, 1978; Neal,1960)

and hepatic infection in hamsters (Jarumilinta, 1966; Mattern and Keister, 1977) and monkeys (Martinez-Reyes et al.,1980). It may be emphasized that there is no universal correlation of virulence observed in animals with the actual extent of the human disease. In particular, a strain of amoeba, H303:NIH, totally avirulent in animals, was isolated from the contents of a liver abscess of a human patient (Diamond et al.,1976). The animal models do, however, provide a semi-quantitative measure of disease potential of specific strains of amoebae, and in studies where different animal models were compared, similar rankings of virulence were generally obtained (Mattern et al.,1978). However, most of the early research in this area was handicapped due to the presence of highly complex micro-flora in its cultures, and more reliable information in this area has emerged with the advent of axenic cultures of **E.histolytica** (Diamond et al.,1978) and development of special animal models which can be infected with such axenic cultures (Ghadirian and Meerovitch, 1978, 1981; Diamond et al.,1978).

ELUCIDATION OF THE ROLE OF BACTERIA IN E.HISTOLYTICA VIRULENCE

Importance of bacterial associates in the invasive mechanism of **E.histolytica** was suspected by several earlier investigators when its axenic cultures were not available. The definitive influence exerted by bacteria in production of amoebic dysentery has since been observed repeatedly (Bracha and Mirelman, 1984; Phillips and Gorstein, 1966; Wittner and Rosenbaum, 1970). Phillips et al. (1955) showed that bacteria were required for the establishment of invasive disease in germ-free guinea pigs infected with amoebae grown with **Trypanosoma cruzi**. They interpreted the role of bacteria

as one providing an environment that enabled amoebae to grow in the colon (Phillips et al.,1958). Wittner and Rosenbaum (1970) demonstrated that heat killed bacteria, crude bacterial homogenates, or media in which bacteria had been grown proved ineffective in causing increased virulence of axenically grown **E.histolytica**. Thus, these results suggested that a factor provided by living bacteria may be required to sustain the virulence of amoebae. Other workers demonstrated that adding pathogenic **Streptococci** to amoebae decreased the incubation period of the experimental disease in kittens and increased the mortality (Phillips et al.,1958). Amoebae grown in vitro which had lost their virulence and were incapable of producing experimental disease recovered their virulence upon addition of bacteria, the most effective being **Escherichia coli**, **Salmonella typhosa** and **Salmonella paratyphi** (Phillips and Gorstein, 1966). These investigators concluded that the type of bacterial associates with which the amoebae are grown in vitro probably constitutes a major factor which determines their infectivity and pathogenicity.

A number of investigators have observed gradual loss of virulence when **E.histolytica** trophozoites is maintained axenically over long durations (Mattern et al.,1982; Phillips, 1973). However, may be regained when the trophozoites are passaged through the hamster liver (Lushbaugh et al.,1978b; Neal and Vincent, 1956). Some pioneering studies which indicated that bacteria could enhance virulence of axenically grown tropozoites of **E.histolytica**, were done by Wittner and Rosenbaum (1970). They found that reassociation of trophozoites with **Escherichia coli** for at least 6 hr before inoculation into the hamster liver dramatically increased the percentage of animals with hepatic abscesses.

Using destruction of tissue culture monolayers by **E.histolytica** as an index of its virulence, Bracha and Mirelman (1984) found that short term association of axenically grown **E.histolytica** trophozoites with bacteria significantly stimulated the amoebic activity. Preincubation of amoebic trophozoites of strain HK-9 for short periods (upto 1 hr) with increasing amounts of an **E.coli** culture that had a surface lectin specific for mannose residues, progressively increased the rate of destruction of monolayers of tissue-cultured mammalian cells. Maximal destruction rates were observed when the ratio of trophozoites/bacteria was 1:1,000, although bacteria alone had no effect on the monolayer of tissue-cultured cells. The addition of α-methyl mannoside to the bacterium-amoeba mixture inhibited the bacterial lectin and prevented the attachment of the bacteria, which thus seems important for bacterial action on amoebic invasiveness.

Increased amoebic virulence was observed after reassociation not only with bacterial strains that possess mannose-binding lectins, but also with types of bacteria such as **Escherichia coli** serotype 055 (Bracha and Mirelman, 1983) that were found to attach to the amoebae by virtue of having cell surface carbohydrates that serve as binding sites for an N-acetyl galactosamine specific lectin present on amoebic cell surface. In these cases N-acetyl galactosamine (also galactose or lactose) inhibited both bacteria-amoeba interaction as well as enhancement of the damage of monolayers by the amoeba (Ravdin et al.,1985c; Ravdin and Guerrant, 1981; Bracha and Mirelman, 1983).

Association of amoebae with bacterial strains which are incapable of attachment or ingestion by the trophozoites (Bracha et al.,1982), such as the anaerobic bacteria **Bacteroides fragilis** and

the gram positive bacteria **Staphylococcus aureus** and **Micrococcus luteus** on the other hand did not stimulate amoebic virulence. A slight augmentation of virulence was observed on association of amoebae with opsonin coated **Shigella** sp., a condition that enabled attachment and ingestion of the bacteria by the trophozoites (Bracha and Mirelman, 1984).

Virulent strains of **E.histolytica** have been shown to contain a variety of soluble toxic substances such as an enterotoxin (Lushbaugh et al.,1979), a variety of collagenase and proteolytic enzymes (Gadassi and Kessler, 1983; Gitler and Mirelman, 1986) and a protein with ionophore forming capabilities (amoebapore) (Lynch et al.,1982). Axenically grown trophozoites which were associated with bacteria for periods of 1 to 2 hr had higher levels of toxic substances (Feingold et al.,1985). This was partially evident in strains with attenuated virulence such as HK-9, in which the level of the enterotoxin after interaction with **E.coli** cells became similar to those present in the highly virulent HM-1:IMSS strain (Mirelman, 1987).

Bacterial contribution in amoebic activities

Trophozoites dwell in the colon in the presence of a variety of bacterial species, and many of these were found to exert a strong chemoattractant effect on the amoebae (Bailey et al.,1985). For many years it was believed that bacteria served as a nutrient source for the amoeba and provided a suitable environment for growth. Bacteria, moreover, were believed to play a role in differentiation of **E.histolytica** i.e. its excystation and encystation and results obtained in recent investigations support this view (Mirelman, 1987). Nakamura (1953) suggested that the associated bacterial flora may

provide an additional enzyme system that the amoebae may require for their metabolism. As anaerobic **E.histolytica** lacks mitochondria, cytochromes, hemo-proteins and catalase but protect itself from highly reactive oxygen metabolites which it may encounter in the aerobic environment prevailing in the host tissues. These reactive products may be injurious to functional elements of the parasite e.g. groups of its vital proteins and membrane lipids/phospholipids etc. Ingested bacteria may protect the amoebae from such toxic effects by removal of hydrogen peroxide through their catalase and may serve as scavengers of toxic free-radicals (Bracha and Mirelman, 1984).

SURFACE FEATURES OF ENTAMOEBA HISTOLYTICA : RELATION TO ITS VIRULENCE

The capacity of **E.histolytica** to move with the help of pseudopodia and ingest host erythrocytes by phagocytosis was recognised by Losch (1875) himself who suggested the presence of a flexible binding membrane in this amoeba. Subsequent investigations indicated that trophozoite's surface membrane may be involved in a variety of functions e.g. endocytosis, pinocytosis, transitional mobility (Jarumilinta and Kradolfer, 1964; Weisman and Korn, 1967; Bowers and Olszewski, 1972), host-cell adhesion and contact dependent invasion leading to eventual engulfment or lysis of host cells (Takeuchi and Phillips, 1975; Ravdin et al.,1980; Ravdin and Guerrant, 1981; Ravdin et al.,1985b).

Transitional mobility of the trophozoites, on account of amoeboid movements and process of pinocytosis and phagocytosis impart distinct characters to the plasma membrane of amoebae and its contractile proteins and actin filaments may have a role in these functions.

Serrano and Reeves (1974, 1975) reported that the entire cell surface of the trophozoites of an **Entamoeba** sp. as well as its isolated plasma membrane has the ability to actively transport glucose. Equilibrative transport of glucose across the membrane was several order higher than that by pinocytosis (Serrano and Reeves, 1975).

Rondanelli et al. (1977) reported an external fuzzy coat (glycocalyx), peripheral to the plasma membrane of the trophozoites. Siddiqui and Rudzinska (1965) noted the presence of a fuzzy layer on the outer surface of the plasmalemma in the electron micrographs of **Entamoeba invadens**. El-Hashimi and Pittman (1970) too, in their electron microscopic studies, observed a similar fuzzy layer in **E. histolytica** trophozoites obtained from colon aspirates of acute amoebic patients and also from monoaxenic cultures. However, they claimed absence of such glycocalyx in case of axenic **E.histolytica**, although its presence was observed by Feria-Velasco et al. (1972) by cytochemical staining. The glycoproteins of the outer layer of glycocalyx may play an important role in the biology of amoeba, particularly in its cellular recognition, adhesiveness and antigenic properties (Feria-Velasco et al.,1972).

Lushbaugh and Miller (1974) using certain histochemical stains viz., alcian blue, periodic acid, shiff's stain (PAS), phosphotungstic acid, colloidal iron and concanavalin A along with horseradish peroxidase, confirmed the acid mucopolysaccharide nature of glycocalyx. They also observed that surface coat of monoaxenically cultured amoebae isolated from intestinal lesions are about twice as thick as those of axenically grown cells. These workers further noticed a thicker deposition of mucopolysaccharide layer on the

amoeba from colon aspirates of acute amoebic patients as compared to the cells in axenic cultures. Whether this fuzzy coat of **E.histolytica** cells is a separate layer unrelated to plasmalemma or just an extension of cell membrane components is still an open question. However, some observations which support the second possibility are given below.

Ito (1965) while studying surface coat of cat intestinal microvilli by cytochemical staining and electron microscopy established its glycan nature. He further reported that this coat presented itself as a continuous layer throughout the inner surface of microvilli and was composed of filamentous protrusion from its epithelial cells, suggesting that this did not exist as an extraneous coat but as an integral part of their plasma membranes. Winzuler (1970) has reviewed in detail the evidence which supports the view that glycocalyx on eukaryotic cell is nothing but an extension of saccharide moieties from globular plasma membrane proteins.

More convincing evidence that surface coat of these cells is an integral part of plasmalemma came from the work of Forstner (1968), who incubated the microvilli epithelial cells with [^{14}C]-glucosamine *in vitro* and recovered the radiolabelled in their plasma membrane fraction after their subcellular fractionation.

In view of the absence of any surface membrane markers of **E.histolytica**, Aley *et al.* (1980) surface-labelled intact **E.histolytica** prior to cell breakage. The labelled cells were exposed to concanavalin A, enabling the surface membrane to be isolated as large sheets resulting presumably in less contamination with intracellular membrane vesicles. These authors failed to find any evidence for a distinct

surface membrane acid phosphatase though they did find a 20 fold enrichment in Ca-ATPase in the surface membrane fraction. While this ATPase was also found in other subcellular fractions, it would appear to be the most authentic surface membrane enzyme activity described so far (McLaughlin and Aley, 1985).

Aley et al. (1980) demonstrated that plasma membrane of **E.histolytica** in SDS-PAGE resolved into twelve iodinated peptides of m.w. from 12-200 kD, all of them giving positive reaction with glycoprotein staining. Cholesterol:Phospholipid molar ratio in the membrane was 0.87. Constituents of Phospholipids were : Phosphatidyl choline, 12, ceramide aminoethylphosphonate, 38, phosphatidyl ethanolamine, 35, phosphatidyl serine, 10 (% of total phospholipids); phosphatidyl inositol and sphingomyelin were present in traces.

Surface lectin receptors in E.histolytica

Since glycosidic linkages of membrane proteins project out of the bilayer and lectins possess specific affinity for different sugars, they have been widely used as probes to elucidate surface features of diverse cells (Sharon and Lis, 1972). A great interest has been developed by the discovery that some lectins can differentially agglutinate viral or carcinogen transformed malignant cells as compared to their normal counterparts (Martinez-Palomo et al., 1972; Burger and Goldberg, 1967). It is also known that some of the virulence related properties of trophozoites participate in adherence (Trissl et al.,1977; Orozco et al.,1980; Kobiler and Mirelman, 1980; Ravdin and Guerrant, 1981). Thus, Trissl et al. (1977) reported that the degree of agglutination induced by concanavalin A (Con A) a lectin isolated from **Concanavalia ensiformis**, which is specific

for glucose and mannose moities, can be used to differentiate pathogenic and non-pathogenic **E.histolytica** strains. Trophozoites agglutination with Con A is due to the presence of membrane mannosylated and glycosylated glycoproteins.

Das (1977) also observed that Con A gave strong agglutination reaction with pathogenic strains of **E.histolytica** while there was little or no reaction with non-pathogenic strains. Pathogenic and non-pathogenic strains of free-living amoebae, however, could not be differentiated on the basis of Con A agglutination since both gave little or no reaction. Prasad et al. (1981) showed that Con A agglutinability was markedly higher in virulent strain of **E.histolytica**, viz. IP:106 as compared to avirulent, DKB strain.

Trissl et al. (1977) observed that treatment of motile forms of **Entamoeba** with low concentration of Con A and subsequently by peroxidase resulted in the formation of clear cut caps of Con A receptors at the posterior pole of the cell. These caps could be separated from the amoebic cells by simple shaking and low speed centrifugation. Treatment with α-methyl mannoside dissociate Con A from the caps and the surface coat sheaths. These observations demonstrate the mobility of Con A receptors in amoebic membrane. Orozco et al. (1980) found that only Con A binds to **E.histolytica** trophozoites, out of several lectins tested. However, strains that differ in virulence viz., HM1:IMSS, HM3:IMSS and HK-9 showed the same ability to agglutinate with Con A as measured by the size of cell clumps formed. Therefore, they suggested that this lectin does not corrlate with amoebic virulence; however, amoebic glycoproteins recognised by Con A still could be involved in the host-parasite relationship.

Prasad et al. (1985) examined agglutinability of three **E. histolytica** strains (DKB, NIH:200 and IP:106) with six other lectins viz. **Ricinus communis** agglutinin and peanut lectin (both galactose specific), **Glycin max** agglutinin (N-acetyl galactosamine specific), wheat germ agglutinin (N-acetyl glucosamine specific) **Limulus polyphemus** lectin (sialic acid specific) and **Lens culinaris** agglutinin (glucose and mannose specific) was investigated. A Ca^{+2} dependent positive response was indicated with the last two which also produced precipitin lines in agar-diffusion test against sodium dodecyl sulphate solubilized sedimentable homogenate fraction. The sugar specificity of **Lens culinaris** lectin is known to be similar to that of Con A and relative agglutination of the three strains by both of them ran parallel to each other. The sialic acid specific lectin was more effective towards the DKB (avirulent strains), whose treatment with trypsin and neuraminidase significantly increased its Con A agglutinability. These results indicate that lower Con A agglutinability of a virulent **E.histolytica** may possibly be due to greater preponderance of sialic acid moities on their surface. Sialic acid is known to be the main contributor of the surface charges in living cells which can possibly retard the agglutination phenomenon. Further, enhancement of Con A agglutination of the avirulent strain by trypsin and neuraminidase treatment indicates that sialoproteins may possibly mask Con A receptors in the avirulent **E.histolytica**. Prasad (1984) following the binding of [^{14}C]-Con A with amoebae noted that the number of Con A receptors on IP:106 was 2.5 times greater as compared to those on DKB strain.

CONTACT MEDIATED KILLING BY E.HISTOLYTICA

Virulent trophozoites can rapidly induce morphological changes in target cell by a contact mediated process. The contact is believed to result in a calcium influx into the amoebae that induces release of a number of proteins at the site of contact. These include the ion-channel forming protein 'amoebapore', which, together with secreted proteases, results in target cell damage and detachment.

Interaction of **E.histolytica** with target host cell involves three sequential steps : (1) adherence by an amoebic adhesion inhibited by N-acetyl galactosamine or galactose residues, (2) cytolysis of target cell (requiring amoebic microfilament function, Ca^{+2} ion flux, role of cytotoxins/enzymes including phospholipase A activity and an acid vesicle pH), and (3) phagocytosis (Ravdin and Guerrant, 1981, 1982 a,b; Ravdin et al.,1980, 1985a, 1986).

Adherence

As with many other enteric pathogens, adherence mechanisms by **E.histolytica** trophozoites appear to have a role in the pathogenesis of disease and form a prerequisite for contact cytolysis of individual target cells (Ravdin et al.,1980; Ravdin and Guerrant,1981).

Takeuchi and Phillips (1975) noted adherence of amoebae to the colonic epithelium prior to their penetration of the interglandular epithelium, a finding confirmed also by Galinda et al. (1975) with colonic organ culture in vitro. Griffin and Juniper (1971) noted amoebae with adherent erythrocytes in colonic biopsies of patients with amoebiasis.

Adherence of amoebae to human erythrocytes has been observed by Trissl et al. (1978) and by Kobiler and Mirelman (1980). Ravdin and Guerrent (1981) noted that this adherence shows greater affinity for human than for bovine or sheep erythrocytes; amoebae adhere equally well to human type A,B or O red cells. Kobiler and Mirelman (1980) isolated a hemagglutinating lectin from **E.histolytica** that was sensitive to inhibition by chitin and other N-acetyl glucosamine containing glycoconjugates. This amoebic lectin is membrane associated, is active at pH 5.7-6.0, heat labile, loses activity at 37°C after 2 hr, not blood group specific and is inhibited by immune IgG.

CanoMancera et al. (1986) analysed the effect of 14 monosaccharides and disaccharides containing galactose (Gal), mannose (Man), glucose (Glc) and some of their analogs on the adhesion of five strains of **E.histolytica** to human red blood cells (RBC) and found that potency of adhesion inhibition were usually higher with carbohydrates of the Gal group, intermediate with Man group, and low with the Glc group. The most potent inhibitor was generally N-acetyl galactosamine.

Ravdin and Guerrant (1981) demonstrated that **E.histolytica** adheres to target Chinese hamster ovary (CHO) cells, which form rosettes around amoebae in media at 4°C with a pH of 6.8. Upon warming to 37°C, the target cells are aggregated in one area (Uroid portion) of the amoebic surface. They also observed that cytochalasins B and D (microfilament inhibitors) block amoebic adherence to target cells at 37°C. This effect of cytochalasin is not due to competitions for the receptor, since no inhibition occurred at 4°C (Ravdin and Guerrant, 1981). Amoebic adherence to target CHO cells and human

RBCs is specifically inhibited by N-acetyl galactosamine (Gal NAc) and galactose (Ravdin and Guerrant, 1981; Petri et al.,1987). Bracha, Kobiler and Mirelman (Bracha et al.,1982; Bracha and Mirelman, 1983) independently confirmed the existance of Gal NAc and galactose inhibitable adherence mechanism of amoeba with baby hamster kidney cells (Bracha and Mirelman, 1984). Bacteria with mannose binding pili adhered to amoebae via mannose receptor present on the surface of **E.histolytica** trophozoites. Certain other bacteria such as **E.coli** strain 055 or **Salmonella greensidi** 50, which were known to contain Gal NAc moities on their lipopolysaccharide (Orskov et al.,1977) while devoid of mannose binding pili, adhered to the trophozoites through interaction with Gal NAc receptor molecules on their surface (Bracha and Mirelman, 1983). Adherence of mannose-binding bacteria occur even with glutaraldehyde fixed or cytochalasin B treated amoebae (Bracha et al.,1982); however, heat inactivation of the bacteria abolished their adherence. Gal NAc sensitive bacterial binding with amoebae, on the other hand was not influenced by heat inactivation or glutaraldehyde fixation although treatment with cytochalasin B prevented the adherence in this case. Bacteria which neither possess Gal NAc, galactose molecules on their surface nor mannose binding pili failed to adhere to the trophozoites. However, when such bacteria were coated with specific antisera (Opsonins) they could bind with the trophozoites. This adherence occurred even if Fab'dimers were used, confirming the lack of Fc like receptors on the amoebae (Ravdin and Guerrent, 1981). Adherence of opsonized bacteria was inhibited by Gal NAc solutions, indicating that the amoebic adhesin recognized such moities on the globulin molecules (Bracha and Mirelman, 1983).

Gal NAc (45 mM) inhibited the lysis of human neutrophils by HMI:IMSS amoebae and enabled the neutrophils to kill these virulent amoebae (Ravdin et al.,1985a). Salata and Ravdin (1986) found that Gal NAc (45 mM) inhibited amoebic destruction of Chang liver cell monolayers and to an even greater extent, the synergistic liver cell destruction observed with both amoebae and neutrophils present.

In summary, these findings demonstrate that Gal NAc specific adherence mechanism of **E.histolytica** is responsible for attachment of amoebae with a variety of cells including certain bacteria, mammalian tissue culture cells, human erythrocytes and leukocytes, mammalian colonic mucosa and fixed human colonic mucosa. This mechanism thus may have an important role in the virulence of **E.histolytica**.

Role of calcium ions in cytolysis

Recent studies on the role of calcium ions in cytolysis of target cells by amoebae demonstrated that amoebic adherence and cytolysis require both the presence of extracellular calcium ions and their intracellular calcium flux (Ravdin et al.,1985a). The calcium is required for : (a) the delivery of lytic substances or toxins, (b) cytoskeletal changes required for mobility or cytolytic activities (chemotaxis, endocytosis, phagocytosis, exocytosis etc.) and (c) action of calcium dependent enzymes, such as those involved in phospholipid metabolism or ion transport, calcium entry into target cells may be required as a final nonspecific mediator of cell death (Long-Krug and Ravdin,1988).

Role of acid-intracellular vesicles in cytolysis

Ravdin et al. (1986) showed that an uninterrupted acid pH in intracellular endocytic vesicles is necessary for the cytolysis of

target cells by **E.histolytica** trophozoites. They observed that NH_4Cl which sufficiently increase vesicle pH, inhibited amoebic killing of target Chinese hamster ovary (CHO) cells. In contrast, NH_4Cl did not affect either the adherence or phagocytic event.

The role of amoebapore (ion channel forming protein) in contact mediated damage of host cell

'Amoebapore' is an amoeba derived protein that is spontaneously incorporated into membranes of the target cell to form ion channels (Lynch et al.,1980; Lynch et al.,1982; Young et al.,1982). Lynch et al. (1982) identified an ion channel forming protein in virulent **E.histolytica** culture. This was associated with high speed sedimentable fraction of **E.histolytica** homogenates and also occurred in the used culture medium, indicating that it may be secreted by the cell during the growth. This amoebapore has the distinctive property of getting spontaneously incorporated in the lipid bilayer, liposomes and cells leading to progressive and irreversible changes in ion conductance of target membranes.

The amoebapore differs from the cytotoxin of **E.histolytica** because it is insensitive to serum and stable at high temperature ($100^{o}C$ for 2 min), whereas it retains full activity if the sample is not boiled (Lynch et al.,1982). This protein is insoluble in triton X-100 which suggests its existence in a highly aggregated form.

Amoebapore is a protein with a monomer molecular weight $14x10^3$ daltons. The monomer is obtained in 0.1% SDS. Treatment of the aggregated intracellular form of amoebapore with 1M NaCl or two cycles of freezing and thawing leads to its dissociation to

a form of $28x10^3$ daltons as determined by gel filtration (Lynch et al.,1982; Young et al.,1982). This shows that SDS can dessociate the aggregated subunits and represents the molecular weight of monomer amoebapore ($14x10^3$ daltons). Further, purification by chromatofocussing and DEAE chromatography showed that there are two forms of amoebapore which have different isoelectric points. About 80% have pI of 6.8 and 20%, 5.3 (Rosenberg, 1985). Both forms can be incorporated spontaneously into artificial lipid bilayers, where they produce single channels (Rosenberg, 1985).

Young et al. (1982) demonstrated that this channel forming protein is released from amoeba after cell surface stimulation with Con A, a calcium ionophore (A-23187) or bacterial lipopolysaccharide.

Role of cytotoxins/enzymes in E.histolytica virulence

Councilman and Lafleur (1891) for the first time reported distruction of intestinal submucosa by the cytolytic action of **E. histolytica** and Dobell (1919) showed that the amoebae in submucosal ulcers in patients were surrounded by clear lytic areas. He proposed that such areas were possibly formed through the action of some cytolytic enzymes released by amoebae.

Neal (1960) and Jarumilinta and Maegraith (1969) reviewed information on the role of **E.histolytica** enzymes in its host tissue invasive process. However, interpretation of most of the earlier work in this area was difficult due to presence of a complex microflora in amoebic cultures.

Neal (1960) demonstrated the proteolytic activity in various strains of **E.histolytica** (both intact amoebae and their extracts) on gelatin and casein. However, the caseinase activity found in

E.histolytica (Neal, 1956) could not be correlated with its virulence since this was found in avirulent strains also.

Further, Jarumilinta and Maegraith (1969) showed both pathogenic and non-pathogenic **E.histolytica** possess tryptic and peptic activity although they were both devoid of chymotryptic activity.

The mechanism by which **E.histolytica** gains a successful entry into the deeper tissues was suspected by Jarumilinta and Maegraith (1960) to possibly depend on the enzymes of the spreading type such as hyaluronidase. However, experimental results of different investigators (Bradin, 1953; Neal, 1960; Jarumilinta and Maegraith, 1962) on this enzyme activity of **E.histolytica** are conflicting and do not provide conclusive evidence of involvement of this enzyme in the invasive mechanism of the amoeba.

Recently, Munoz et al. (1982) and Gadasi and Kessler (1983) reported presence of collagenase in **E.histolytica** which existed at a markedly higher level in its virulent strains. A close correlation between the ability to produce liver lesions in hamster and collagenolytic activity of various strains of **E.histolytica** has been found by Munoz et al. (1984).

Munoz et al. (1982) described a collagenase, with greater activity on type I collagen, which produced specific cleavage products (75,000, 50,000, 25,000 molecular wt.) during short incubation with the substrate. Like mammalian collagenases, the amoeba enzyme causing the initial digestion of collagen was not inhibited by N-methyl maleimide, cysteine inhibited rather than activated, the reaction was observed with serum or EDTA. The collagenolytic activity of **E. histolytica** reported by Gadasi and Kessler (1983) corresponds to the

enzyme described by Munoz et al. (1982) that completed degradation of collagenase cleavage fragments during longer incubation periods. Collegenolytic activity was enhanced by dithiothreitol and inhibited by N-ethyl maleimide. These finding suggest the presence of atleast two amebial enzymes (proteases) acting on collagen, a specific collagenase and a non-specific collagenolytic enzyme.

McLaughlin and Faubert (1977) were the first to study proteolytic enzymes in an axenically cultivated strain of **E.histolytica**. They reported and partially purified an acid proteinase resembling cathepsin D and a neutral sulfhydryl proteinase resembling cathepsin B. The acid proteinase, with a mw of about 41,000 and a pH optimum near 3.5, acted on haemoglobin and human serum albumin. The neutral proteinase failed to affect either of these substrates at a low pH but had slight asctivity at pH 6.0. This neutral proteinase, mw 27,000, exhibited more activity on azocasein at pH 6.0-6.3.

Lushbaugh et al. (1978a) showed that cell free extracts prepared from washed trophozoites grown in axenic culture caused cytopathogenic effects on cell cultures if no serum was present. Bos (1979) proposed a correlation between the virulence of **E.histolytica** trophozoites and their cytopathic effects. Lushbaugh et al. (1979) found the cytotoxic activity to be associated with a fraction containing proteins with mw of about 30,000. Bos (1979) isolated and characterized an intracellular toxin from cell free extracts of axenically cultivated HK-9 tropozoites. They suggested that the same **E.histolytica** factor may be responsible for contact dependent lysis and toxin induced cytopathic effect and pointed out several similarities between the amoebic 'cytotoxin' and the low m.w.

thiol proteinase described by McLaughlin and Faubert (1977). McGowan et al. (1982) compared the cytotoxic activity of extracts of four strains of **E.histolytica** which differed in their virulence. Crude extracts of virulent strain HM-1 exhibited much greater cytotoxic activity than that by a less virulent strain 200 or Rahman and 303.

The concept of several amoebae proteinases acting together to hydrolyze neutral substrates was further explored by Gadasi and Kobiler (1983). They demonstrated that crude extracts of virulent HM-1 had 32 times higher proteolytic activity per mg of protein on azocol than did HK-9. They suggested that proteolytic enzymes are involved in the detachment of tissue culture cells from their substratum and are thus responsible for the cell-rounding cytopathogenic effect of amoebic 'cytotoxin' in vitro. The content of proteolytic enzymes found in a given strain thus may bear a correlation with amoebic capacity to penetrate into the tissue and hence its virulence.

Later, Lushbaugh et al. (1984b) attempted separation of the cytotoxic activity from the proteinase activity of amoebic cytotoxin using chromatography and isoelectric focussing technique. Some high m.w. fractions without proteolytic activity had cytotoxic effects as did certain low m.w. fractions with basic isoelectric points. One of the high m.w. fraction with some cytotoxic activity may be amoebic cathepsin D. The major cytotoxin isolated was a thiol proteinase (m.w. 20,000) with an isoelectric point between pH 4 and 5, that may be amoebic cathepsin B.

A weekly 'acidic protease' with "powerful proteolytic activity" has recently been isolated from trophozoites of virulent strains HM-1 of **E.histolytica** by Scholze and Werries (1984). This enzyme had

maximal activity on azocasein at pH 4.4, but was also active at pH 3.4 and 8.5. Lushbaugh et al. (1985) purified the cathepsin B of **E.histolytica** using ion exchange chromatography and agarose affinity chromatography. The purified enzyme (m.w. 16,000) had proteinase activity that could be demonstrated on azocasein and haemoglobin. The virulent strains of **E.histolytica** (HM-1 and Rahman) had significantly higher cathepsin B activity on carbobenzoxy-L-arginyl-7-amino-L-trifluoramethyl coumarin, a substrate specific for cathepsin B per mg protein than less virulent strains (HK-9, Laredo and Huff).

Another enzyme which possibly may be involved in the invasive function of **E.histolytica** is acid phosphatase which was reported for the first time in the year of 1948 by Carrera and Changus (1948). Eaton et al. (1970) and Trevino et al. (1971), showed high acid phosphatase activity in both 'surface active' and cytoplasmic 'lysosomes', food vacuoles and also in the intracellular bodies. Eaton et al. (1969,1970) and Feria-Velasco and Trevino (1972) proposed that this enzyme may be associated with cupshaped surface lysosomes and play an important role in the tissue invasive function of **E.histolytica**. More recent results suggest that acid phosphatase may be associated both with plasma and phagosomal membranes of **E.histolytica** (Aley et al.,1980, Warren et al.,1982). Pandey et al. (1977) studied both alkaline and acid phosphatases in the crude homogenate of axenic **E.histolytica** and reported markedly higher activity of acid phosphatase as compared to alkaline phosphatase.

A number of acid hydrolases (found generally in lysosomes) other than acid phosphatase have been found to be associated with sedimentable structures in **E.histolytica**. These include phosphatase

(McLaughlin and Merrovitch, 1975b; Serrano et al.,1977; Van Vliet et al.,1976), esterase (McLaughlen and Merrovitch, 1975b), proteinases (McLaughlin and Faubert, 1977) and RNAse (Weller et al.,1981). McLaughlin and Merrovitch (1975a) demonstrated that these hydrolases were contained in sedimentable structures, displayed latency, and phosphatase and N-acetyl-glucosaminidase at least, were not released by repeated cycles of freezing and thawing. Katiyar et al. (1988) recently reported higher basal levels of acid hydrolases viz., phosphatases, ribonucleases (RNAse), deoxyribonucleases (DNAse) and proteinases in virulent (IP:106) strain as compared to NIH:200 strain of **E.histolytica.**

Role of amoebic phospholipases in cytolysis of target cells

Phospholipid metabolism has been shown to be linked with lytic processes in **E.histolytica** (Ravdin et al.,1985a) and bacteria (Doery et al.,1965, Liu, 1974). Possible involvement of phospholipases in virulence of **E.histolytica** is indicated from the observations suggesting its role in the haemolytic activity of its homogenates (Said-Fernandes and Lopez-Revilla (1982) and the cytotoxic effect of trophozoites on cellular monolayers (McLaughlin and Aley, 1985). Amoebic phospholipases may damage cells by : (1) interacting directly with phospholipid constituents of target cell membrane, (2) producing lytic substances as by products of the hydrolysis of amoebic or target cell phospholipids.

Entamoeba membranes consist predominantly of five glycerophospholipids, phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol, sphingomyelin and ceramide aminoethylphosphonate (Aley et al.,1980; McLaughlin and Meerovitch, 1975b) . Presence of phospholipid, ceramide aminoethylphosphonate is uncommon in other

eukaryotic cells (Kittredge and Roberts, 1969). These phospholipids enhance membrane stability in hydrolytic environments in gut because of phosphodiester bond (Kittredge and Roberts, 1969).

Amoebae have been shown to contain phospholipases A and C, lysophospholipase and sphingomyelinase enzymes (Long-Krug et al., 1985; Aley et al.,1980; Ravdin et al.,1985a). The role of phospholipase C in cytotoxicity remains to be determined. Long-Krug et al. (1985) studied sub-cellular localization of phospholipase A in the trophozoites of **E.histolytica** strain IMSS. They reported two phospholipase A enzymes of **E.histolytica**. One is calcium independent and optimally active at acidic pH (4.5) and the other is calcium dependent and most active at alkaline pH (7.3). Calcium dependent phospholipase A activity was localized in fractions derived from the plasma membrane while calcium independent phospholipase A activity was predominantly localized in soluble sub-cellular fractions.

Ravdin et al. (1985a) postulated the role of calcium as a second messenger leading to the release of cytotoxic lyso-compounds produced by amoebic phospholipase A. A putative antagonist of intracellular calcium flux, 9-(N,N-diethyl amino) acetyl 3,4,5-trimethoxy benzoate (TMB-8, 250 uM) and calcium chelators EDTA (10 mM) and EGTA (10 mM) inhibited cytolysis of the target cells. Hence, these workers concluded that extracellular calcium ions, ameobic intracellular calcium flux and amoebic phospholipase A activity are required in this invasive function of **E.histolytica**. Amoebic phospholipase A may alter the integrity of target cell membrane and render the target cell permeable to attack by other amoebic enzymes (Ravdin et al., 1985a).

Contact mediated phagocytosis

Phagocytosis is a property of **E.histolytica** that not only correlates with virulence (Trissl et al.,1978; Orozco et al.,1980; Orozco et al.,1982) but has been demonstrated to be involved in the pathogenic mechanism of trophozoites. The role of phagocytosis in **E.histolytica** pathogenicity became evident by using a genetic strategy that included the isolation of phagocytosis deficient amoebic mutants that showed a deficiency in the in vivo virulence (Orozco et al.,1983).

Entamoeba have the ability to phagocytose a variety of particulate materials including starch grains, bacteria, various protozoa, inert polystyrene beads and erythrocytes (Lushbaugh et al.,1976; Neal, 1966; Shaffer and Balsom, 1954). More than hundred years ago Losch (Losch, 1875) demonstrated that amoebae in stool samples during acute stages of human dysentary contained large number of erythrocytes. Since then it has been claimed repeatedly that only invasive strains of **E.histolytica** inhabiting human intestine ingest erythrocytes and erythrophagocytosis has been traditionally considered as an important criteria in identifying pathogenic **E.histolytica** trophozoites. On the other hand **Entamoeba** other than **E.histolytica** have occasionally been shown to engulf erythrocytes both in vivo and in vitro (Zaman, 1970; Lynch, 1924; Tyzzer and Quentin, 1938).

Trissl et al. (1978) showed significant differences in the erythrophagocytotic ability of different **E.histolytica** strains cultivated axenically. Thus pathogenic strains were found to phagocytose relatively higher number of erythrocytes as compared to non-pathogenic ones. This rate may be determined by the number and affinity of specific adhesins and other receptors capable of interacting with the target cell. Evidence for this has been obtained from experiments using

adhesion deficient clones (Garcia-Rivera et al.,1982), by which it was demonstrated that impairment in adhesion resulted in both a lower rate of phagocytosis and a decrease of the in vitro and in vivo aggressiveness of trophozoites.

The plasma membrane of **E.histolytica** is covered with some of the parasite antigens that particpate in the initial contact with target cells during the phagocytosis. These antigens contain exposed mannose or glucose residues, since they bind Con-A, which interact specifically with those sugars (Martinez-Palomo et al.,1973). Electron microscopic studies have shown variation in the thickness of the surface coat of **E.histolytica** trophozoites, depending on the origin of the amoeba (Martinez-Palomo, 1982). Surface coats have been reported to be most prominent in invasive trophozoites isolated from colonic lesions or liver abscesses, compared to the less virulent axenic amoebae (El-Hashimi and Pittman, 1970; Proctor, 1976).

An evidence for the participation of microfilaments in **E. histolytica** phagocytosis was given by Ravdin et al. (1980). These authors reported that reduction of motility and inhibition of both phagocytosis and cell distruction occurs after incubation of trophozoites with cytochalasin B and D. Compounds such as colchicine and vinblastine, which depolymerize cytoplasmic microtubules, do not inhibit phagocytosis (Ravdin et al.,1980), suggesting that tubulin does not participate directly in phagocytosis.

ROLE OF CHOLESTEROL IN E.HISTOLYTICA VIRULENCE

Polyaxenic as well as axenic cultures of amoeba generally lose their virulence or invasive property on prolonged cultivation in vitro (Phillips, 1973), when tested in terms of their lesion-producing

ability in the liver of hamsters or in the caeca of rats and guinea pigs. However, such attenuated cultures may regain this function on in vitro passage in host livers.

Singh (1959), Sharma (1959) and Singh et al. (1971) for the first time reported that avirulent strains of **E.histolytica** became invasive when their bacteria associated cultures were supplemented with cholesterol or when animals were fed with cholesterol along with their normal diet before being intracaecally inoculated with non-invasive amoeba. This finding has since been confirmed by numerous other investigators, who demonstrated that the virulence enhancing action of cholesterol occurs also in axenic cultures (Das and Ghoshal, 1976; Vinayak et al.,1978; Meerovitch and Ghadirian, 1978a). Thus Bos and Van de Griend (1977) working with two axenic strains HK-9 and HB-301 were able to restore the virulence with cholesterol in vitro and establish a higher degree of virulence also by two successive passages through hamster liver. They further observed that epicholesterol, the 3 α-hydroxy isomer of cholesterol, which unlike cholesterol does not induce changes in the permeability of the cell, had the same effect.

Meerovitch and Ghadirian (1978 a,b) demonstrated revival of the lost pathogenicity of three strains of **E.histolytica**, HM-1: IMSS and DKB (growing axenically for the last five and six years, respectively) through a number of successive transfer in culture medium supplemented with extra-fine cholesterol. The number of passages in cholesterol-enriched medium necessary to restore a certain degree of pathogenicity of these strains in hamsters, was proportional to the time of in vitro cultivation of the strain and not just the time

of cultivation, under axenic conditions. Pathogenicity, once restored persisted for a long time after cholesterol treatment was stopped.

Mechanism of cholesterol action

Parallel to enhancement of **E.histolytica** virulence in terms of its lesion forming ability in animals, potencies or levels of a number of biochemical factors (which are quantitatively higher in virulent strains) have been found to increase on cultivation of the amoebae in cholesterol enriched environment. Some of these parameters are listed below :

1. Concanavalin A agglutinability (Prasad et al.,1981; Katiyar et al.,1987).
2. Haemolytic activity (Prasad et al.,1982; Katiyar et al.,1988, 1989).
3. Activity of 'lysosomal' and certain other enzymes, viz. acid RNAse acid DNAse, acid phosphatase, acid protinease, phosphoglucomutase, hexokinase, phospholipase A and sphingomylenase (Rai et al.,1980; Lal and Garg, 1979; Lal et al.,1980; Dutta et al.,1982; Katiyar et al.,1987; Katiyar et al.,1989; Mishra et al.,1988).

The precise mechanism of virulence enhancement of **E.histolytica** by cholesterol, however, is still unclear, although a number of hypotheses have been put forward in this context. Rothman and Eagelman (1972) demonstrated that cholesterol exerts different effect on the fluidity of synthetic membranes constituted of lipids/phospholipids at higher and lower temperatures. It has been proposed that the levels of cholesterol in amoebae may thus affect the structure and

function of the plasma membrane of the trophozoites and facilitate the release of the lysosomal enzymes from the 'surface-active lysosome' situated at the tip of filopodia of **E.histolytica** (Eaton et al.,1970; Proctor and Gregory, 1972; Lushbaugh et al.,1978a; Katiyar et al.,1988).

Several early workers (Synder and Meleney, 1943; Rees et al., 1944; Hansen and Anderson, 1948) demonstrated that cholesterol is a growth promoting factor in **E.histolytia** and this property of the sterol has also been considered a possible factor in its virulence enhancing action .

Chen et al. (1975) suggested that a period of enhanced cholesterol synthesis is pre-requisite for the subsequent phase of DNA synthesis and for the completion of mitotic cycle in lymphocytes after phytohaemagglutination activation. Kandutsch and Chen (1977) showed that DNA synthesis showed a linear decline soon after the addition of inhibitors of sterol biosynthesis. Hradec and Dusek (1968) and Hradec et al. (1971) reported that cholesterol may play an important role in some of the reactions involved in cholesterol's ability to stimulate such anabolic functions of cells. This has been regarded as one of the possible mechanisms whereby a general increase in acid hydrolases may occur in **E.histolytica** (Lal and Garg, 1979). However, recent observations of Katiyar et al. (1988) showed that the sterol does not bring about any significant change in the activities of **E.histolytica** alkaline hydrolases. They also noted that although hexokinase and phosphoglucomutase were enhanced by high medium cholesterol, glucose phosphate isomerase maintained itself at its basal level. This seems to exclude the possibility that observed enzyme changes are due to general modulation of protein synthesis. Such

effects moreover should be uniform for all proteins and should not cause much change in enzyme specific activity values.

Martinez-Palomo (1982) proposed that the mechanism of virulence escalating action of cholesterol may comprise of selection of pre-existing virulent sub-populations in the cultures. The heterogenicity of **E.histolytica** cultures was indicated from their observations that poisonous bacteria induce significant reduction in culture virulence. This hypothesis has found support from recent observations of Katiyar et al. (1989), who observed that cholesterol enriched medium resulted in marked enhancement of haemolytic potency and activities of acid hydrolase in a parent avirulent culture (DKB), however, such changes did not occur in the clonal cultures derived from the same strain by similar cholesterol treatment. The same investigators inferred that all these data are apparently in agreement with the concept that high cholesterol may be instrumental in selecting out certain sub-populations in a heterogeneous mixture of genetic variants (Martinez-Palomo, 1982; Das et al.,1982), this being particularly true for strain showing low virulence.

HAEMOLYTIC ACTIVITY OF E.HISTOLYTICA AND VIRULENCE

Lopez-Revilla and Said-Fernandez (1980) devised an in vitro assay to correlate the haemolytic activity of cell free extracts of **E.histolytica** trophozoites or certain other amoebae with their tissue necrotic activity with the help of an end point titre method. The four species tested included **E.histolytica**, **E.invadens**, **E.moshkovskii** and **Laredo** type of **E.histolytica**, and the activity was found to be highest in **E.histolytica**. Further, the potency of activity varied in different strains of **E.histolytica** and was highest in the most

virulent strain (HM-3:IMSS) followed by HM-2:IMSS (moderately virulent) and least in HK-9 (avirulent). Prasad et al. (1982) similarly showed that haemolytic potency was highest for the virulent strain IP:106 followed by moderately virulent NIH:200 and was least in the avirulent strain (DKB).

Lopez-Revilla and Said-Fernandez (1980) reported that this haemolytic effect was more specific for rodent that human red blood cells. Haemolytic activity was localized in a vesicular fraction and was maximal at pH 8 in the presence of 1.0 mM Ca^{+2}, and was lost by heating to 90°C or repeated freezing-thawing (Said-Fernandez and Lopez-Revilla, 1982).

Possible involvement of phospholipases in the virulence of **E.histolytica** is indicated from the observations suggesting its role in the haemolytic activity of its homogenates (McLaughlin and Aley,1985).

Said-Fernandez and Lopez-Revilla (1988) have recently reported that free fatty acids released from phospholipids are major heat stable haemolytic factors of **E.histolytica** trophozoites.

ZYMODEME PATTERN OF E.HISTOLYTICA AND VIRULENCE

A zymodeme is a population of an organism showing a characteristic electrophoretic pattern of certain specified enzymes. Distinctions between isoenzyme patterns reflected in their electrophoretic mobilities have been exploited as a method of characterizing strains of a variety of parasites e.g. Plasmodium, Trypanosoma and Leishmania etc. Isoenzymic patterns have been claimed to be specific and stable properties of strains. Reeves and Bischoff (1968) for the first time examined electrophoretic behaviour of five amoebic enzymes. viz., glucokinase, phospho-

glucomutase, glucose phosphate isomerase, malate dehydrogenase and NADP diaphorase.

Sargeaunt and colleagues (Sargeaunt and Williams, 1978; Sargeaunt et al.,1980) showed that different isolates of **Entamoeba** (from the stool of patients) can be characterized in terms of electrophoretic profile of certain isoenzymes. All these isolates exhibited similar migration of malate dehydrogenase but the different pattern of hexokinase, glucose phosphate isomerase and phosphoglucomutase could be used to classify these isolates into several distinct groups (Zymodemes). The more common zymodemes I and III were always associated with commensal amoebae derived from asymptomatic carriers with apparently negative serology, while zymodeme II was only found in isolates from symptomatic individuals (Sargeaunt and Williams, 1982). A significant number of other minor patterns were also found. However, in every case the pattern of zymodeme of asymptomatic cases (non-pathogenic zymodemes) were distinct from those observed in isolate from individuals with disease (pathogenic zymodemes). Thus, they claimed identification of as many as 22 zymodemes of **E.histolytica** (Sargeaunt et al.,1982 a,b,c). All **E.histolytica** strains isolated from clinical amoebiasis patients were found to belong to any one of the following seven zymodemes: II, VI, VII, XI, XII, XIII, XIV. Further, they claimed that zymodeme XIV was the sole pathogenic zymodeme in the Indian subcontinent (Sargeaunt et al.,1984).

No evidence of alteration in isozyme patterns i.e. shift from non-pathogenic to pathogenic profile or vice-versa, was ever demonstrated in longitudinal culture studies till recently (Sargeaunt, 1985; Sargeaunt et al.,1982 d). However, Mirelman et al. (1986a) demonstrated that the isoenzyme electrophoretic pattern of an axenic non-pathogenic

CDC strain 0784:4 of **E.histolytica** changes from a non-pathogenic to pathogenic pattern when the nutritional supplement consisting of irradiated bacterial cells were provided in the culture medium. This culture retains newly acquired pathogenic zymodeme after continuous treatment with a variety of antibiotica which finally yielded an axenic culture.

Two possible explanations were offered for the observed shifts to pathogenic zymodeme obtained with above **E.histolytica** strain (Mirelman et al.,1986a). Thus, such a change could be ascribed either to induction of an altered isoenzymic pattern in the culture by the conditions of the experiment or alternatively selection of pre-existing sub-population of the new zymodeme under growth conditions employed. To exclude the latter possibility, Mirelman et al. (1986b) conducted similar experiments using a clonal culture of a non-pathogenic strain (SAW 1734 R cl AR, zymodeme group III). The results apparently confirms that an amoeba possessing a characteristic non-pathogenic isoenzymic profile can change to 'pathogenic' one by specific growth conditions. It may, however, be pointed out that the clonal culture employed by Mirelman was not a freshly isolated and thus subject to having developed mixed sub-populations during the course of its maintenance. In any case these recent experiments of Mirelman et al. (1986b) cast doubt on the earlier belief that the isoenzyme profile constitues a stable virulence related property of any culture.

REDUCING/OXIDISING POTENTIAL OF E.HISTOLYTICA AND ITS VIRULENCE

Bracha and Mirelman (1984) on the basis of the studies using disruption of baby hamster kidney cell monolayers as an index of their virulence proposed that virulence of a given **E.histolytica** strain may depend on the activity of its electron transport system

or the cell's 'reducing power'. Both anaerobic conditions and ingested bacteria (especially aerobic ones which have surface features to support their phagocytosis) apparently favour a reduced state of the amoebic cell; the first through oxygen deprival (Montalvo et al.,1971) and the latter by acting as broad-range scavangers of toxic oxidised molecules or radicals. Iron binding proteins of the bacteria such as enthrochelins (Konisky, 1979) and cytochromes as well as proteins rich in sulfhydryl groups may particularly serve as electron donors and contribute in maintaining the reduced state of the trophozoites.

The amoebic drug metronidazole is apparently reduced to form a highly active entity through the mediation of amoebic ferredoxin molecules, and this may subsequently inhibit amoebic macromolecular synthesis (Muller, 1983). Bracha and Mirelman (1984) reported that the agents that specifically consume the reducing power of the trophozoites, such as metronidazole which reduce ferredoxin, drastically inhibited the amoebic virulence both under anaerobic conditions and in bacteria stimulated trophozoites. However, the uptake of [^{14}C]-metronidazole by trophozoites of **E.histolytica** was markedly enhanced in the presence of bacteria. Since the uptake of metronidazole molecules by trophozoites is known to be a function of their rate of reduction by the ferredoxin, the increased in vitro uptake, in the presence of bacteria was a clear indication of an acceleration in the cell's electron transport system or an increase in the reducing power of the cell (Mirelman, 1987).

Aust-Kettis et al. (1982) on the other hand, observed that phagocytosis of heat killed bacteria or exposure of antiamoebic antibodies, stimulated nitro blue tetrazolium (NBT) reducing ability of

E.histolytica. This may attributed to increased production of reactive oxygen intermediates as occurs in mallalian phagocytes, a phenomenon designated as respiratory burst (Filippo, 1986). These results suggest that both oxidizing and reducing potentials generated during this respiratory activity of **E.histolytica** may berelated to its pathogenic ability.

PYROPHOSPHATE METABOLISM IN E.HISTOLYTICA

Chemotherapy requires selective elimination of the pathogenic organism in its host without causing any serious enjury to the latter. This presumably is possible only if the metabolism/cellular organization has some functionally important distinctions between the two, which may provide selective targets for being attacked by the drug. Identification of such specific features can thus be useful in designing chemotherapeutic strategies.

Inorganic pyrophosphate (PPi) is produced as a byproduct during a number of anaerobic cell processes, such as fatty acid synthesis and amino acid activation during synthesis. This PPi has to be removed to shift the reaction equilibrium in favour of synthesis. In most cells a Mg^{+2} dependent pyrophosphatase (PPiase) maintains intracellular PPi concentrations at levels compatible with the functioning of these synthetic processes. However, **E.histolytica**, like certain bacteria (Klemme, 1976; Wood et al.,1977) is able to make use of the energy present in PPi molecule rather than wasting it by the hydrolytic formation of Pi. This amoeba has been shown to possess considerably higher levels ($2x10^{-4}$ M) of PPi in its cytosol (Reeves et al.,1974). A major source of this PPi (Wood et al.,1977) is believed

to be carboxytransphosphorylase catalysed formation of oxaloacetate from phosphoenolpyruvate (Fig.A).

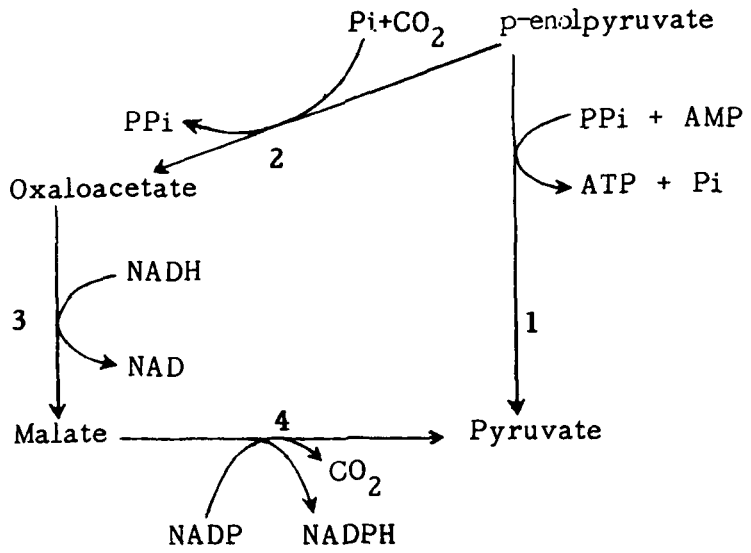


Fig.A : Pathways for pyruvate formation from phosphoenolpyruvate, 1 = pyruvate phosphate dikinase; 2 = phosphoenolpyruvate carboxytransphosphorylase; 3 = malic dehydrogenase; 4 = malic enzyme.

Further, the enzyme acetate kinase (Reeves and Guthrie, 1975) which catalyses formation of acetylphosphate and orthophosphate from acetate and PPi with a favourable equilibrium in rightward direction may constitute another unique source of PPi formation in **E.histolytica**, which may utilise acetyl phosphate generated by gut dwelling bacteria.

$$\text{Acetyl phosphate} + \text{Pi} \underset{\text{Acetate kinase}}{\rightleftharpoons} \text{Acetate} + \text{PPi}$$

As mentioned, regulation of cellular pyrophosphate levels may be necessary for the maintenance of required rate of fatty acid synthesis, amino acid activation and mechanisms which bring about disposal/utilizations of PPi also attain particular functional significance

in this system. The mentioned cytosolic PPiase involved in the disposal of pyrophosphate in higher organisms is apparently absent in **E.histolytica** (although a high Km membrane bound PPiase has been reported, McLaughlin et al.,1978) and the PPi produced in the amoeba is believed to be utilised largely by unique enzyme reactions viz. PPi-dependent phosphofructokinase (PPi-PFK) and pyruvate phosphate dikinase (PPD). PPi-PFK catalyses the reaction:

$$\text{Fructose 6-PO}_4 + \text{PPi} \rightleftharpoons \text{Fructose 1-6,diPO}_4 + \text{Pi}$$

This enzyme thus carries out the function of ATP dependent phosphofructokinase with net saving of ATP through utilizing the phosphoanhydride energy of PPi (Reeves et al.,1976). The above reaction is reversible and unlike mammalian ATP-dependent PFK, the amoebal PPi-PFK has a m.w. of 83,000 (m.w. of mammalian ATP-PFK:3,20,000) with absolute requirement of divalent metals (optimally Mg^{+2}). Further, this enzyme in **E.histolytica** seems to have no role in regulating glycolytic flux (Reeves et al.,1976).

Another PPi dependent enzyme is pyruvate phosphate dikinase, which catalyses the reaction:

$$\text{Pyruvate} + \text{ATP} + \text{Pi} \rightleftharpoons \text{p-enolpyruvate} + \text{AMP} + \text{PPi}$$

The equilibrium of this reaction being towards the left, thus seems to fulfill the glycolytic role normally assigned to pyruvate kinase of the host cell with concomitant synthesis of ATP at the cost of PPi (Reeves, 1968).

These unique PPi metabolising enzymes may possibly provide suitable targets for chemotherapy of amoebiasis and some studies on pyruvate phosphate dikinase of **E.histolytica** which were undertaken with this objective in view are reported in this dissertation.

PLAN AND SCOPE OF THE WORK

The main objectives of the research reported in this dissertation were:

1. To elucidate the physiological/biochemical factors related to invasive action of the **E.histolytica** on host tissue.
2. To elucidate the role of cholesterol in the modulation of amoebic virulence.
3. To identify specific biochemical features of **E.histolytica** as potential target for selective chemotherapy.

To study employed axenic NIH:200 and IP:106 strains of **E.histolytica** which show marked differences in their invasive properties. Further, the former culture (NIH:200), passaged more than 20 times through cholesterol enriched medium, was also included in the study. Studies of erythrophagocytosis employed also xenic DKB strain (which shows very low virulence) and its clonal cultures derived from the micromanipulative isolation and cultivation of single cell.

The parameters investigated include erythrophagocytosis, CO_2 production, concanavalin A internalization, NBT reduction, oxygen uptake, alcohol dehydrogenase, intracellular and extracellular acid phosphatase, cysteine proteinase and pyruvate phosphate dikinase activities in this amoeba. Comparative levels of these parameters in the trophozoites of specified **E.histolytica** cultures and their salient properties including sensitivity to specific inhibitors/activators were also investigated.

CHAPTER II

MATERIALS AND METHODS

SOURCE OF ENTAMOEBA HISTOLYTICA CULTURES USED IN THE STUDY

Three strains of **E.histolytica** viz. NIH:200, IP:106 and DKB were used in the study, which were received from Dr. S.R. Das, Division of Microbiology, Central Drug Research Institute, Lucknow. The strain NIH:200 was obtained by him from Dr. L.S. Diamond, National Institute of Health, MD, USA, whereas other two strains, DKB and IP:106 from Dr. E. Meerovitch, McGill University, Quebec, Canada.

MAINTENANCE OF E.HISTOLYTICA CULTURES

The axenic NIH:200 and IP:106 strains were maintained in RNA supplemented modified Diamond's TP-S-II (Trypticase-Panmede-serum) medium (Diamond, 1968). The modification was based on research undertaken at CDRI (Imam, 1986). The DKB strain of **E.histolytica** was maintained in Diamond's TYI-S-33 (Trypticase-yeast extract-iron serum) medium (Diamond et al.,1978). DKB strain, having developed some bacterial contaminants was maintained and grown in the presence of streptomycin (500 $\mu g\ ml^{-1}$) penicillin (500 $ug\ ml^{-1}$) and terramycin (1.25 $ug\ ml^{-1}$), which almost completely suppressed the growth of the contaminants. Both the above

these media were constituted by mixing a number of solutions whose composition and preparation procedure is described below:

COMPOSITION OF THE NUTRIENT BROTH FOR TPS-II MEDIUM

(Diamond, 1968; Imam, 1986)

Trypticase (BBL)	10.0 g
Panmede (Paines and Byrne)	20.0 g
Glucose	5.0 g
L-Cysteine (monohydrochloride)	2.0 g
Sodium chloride	5.0 g
Potassium phosphate (monobasic)	0.6 g
Potassium phosphate (dibasic)	1.0 g
Ribonucleic acid	2.5 g
Distilled water	895.0 ml

The nutrient broth was suction filtered in Buchner funnel through Whatman No.1 filper paper, then autoclaved at 15 lbs pressure (121°C) for 15 min and allowed to cool at room temperature. pH was adjusted to 7.0 with 1N NaOH (before autoclaving).

VITAMIN MIXTURE NO.107 FOR TPS-II

Vitamin mixture No.107 of Evans et al. (1956) was used, which was prepared from five stock solutions as follows:

1. **Water soluble B vitamins**

Solution A

Niacin	62.5 mg
Para-aminobenzoic acid (PABA)	125.0 mg

These were dissolved in boiling water and final volume brought to 150 ml with distilled water.

Solution B

Niacinamide	62.5 mg
Pyridoxine hydrochloride	62.5 mg
Thiamine hydrochloride	25.0 mg
Calcium pantothenate	125.0 mg
Inositol	125.0 mg
Choline chloride	1250.0 mg

These were dissolved in distilled water and final volume made to 150 ml.

Solution C

Twenty five mg riboflavin was dissolved in 75.0 ml distilled water with the help of 0.1N NaOH, drop by drop and final volume brought to 100 ml with distilled water.

Solutions A,B and C were combined and final volume was made up to 500 ml with distilled water.

2. **Biotin solution**

Thirty mg D-biotin was dissolved in 200 ml distilled water with the help of 0.1N NaOH, drop by drop and final volume brought to 300 ml with distilled water.

3. **Folic acid solution**

Thirty mg folic acid was dissolved in 200 ml distilled water with the help of 0.1N NaOH and final volume brought to 300 ml with distilled water.

4. **Lipid soluble vitamins**

Solution A

Three hundred mg vitamin D_2 (calciferol) was dissolved in 63.0 ml of 95% (v/v) ethyl alcohol. Then 300 mg vitamin A was added to this solution and mixed.

Solution B

Sixty mg vitamin K (menadione sodium bisulphate) was dissolved in 300 ml of 5% aqueous solution of tween-80 .

5. **Vitamin E solution**

Twenty five mg vitamin E (α-tocopherol acetate) was dissolved in 250 ml of ethyl alcohol.

To prepare final working solution, these five stock solutions were combined as follows :

Solution A,B and C combination	500 ml
Biotin solution	250 ml
Folic acid solution	250 ml
Lipid soluble vitamins	2,500 ml
Vitamin E solution	250 ml

This complete mixture was sterilized by passing through seitz filter and then stored at $-20^{\circ}C$ until used.

Antibiotic solutions

1. Streptomycin 1.0 gm dissolved in 5.0 ml sterile distilled water.
2. Penicillin (10,00,000 units) was dissolved in 5.0 ml sterile distilled water
3. Terramycin (injectable) 0.1 ml was dissolved in 20.0 ml sterile distilled water.

Serum-vitamin mixture

Eighty ml of inactivated (56°C for 30 min) adult buffalo serum was mixed with 20.0 ml of vitamin mixture prepared as described above.

This serum - vitamin mixture was sterilized by passing through Seitz filter.

Complete TP-S-II medium

The constituents of complete TP-S-II medium for 100 are:

Autoclaved TP-S-II nutrient broth	85.0 ml
Sterilized serum vitamin mixture	15.0 ml
Penicillin	0.25 ml
Streptomycin	0.25 ml
Terramycin	0.25 ml

Aseptic conditions were maintained throughout the preparation of medium.

COMPOSITION OF NUTRIENT BROTH FOR TYI-S-33 MEDIUM

(Diamond et al., 1978)

Trypticase (BBL)	20.0 g
Yeast extract (BBL)	10.0 g
Glucose	10.0 g
Sodium chloride	2.0 g
Potassium phosphate (monobasic)	1.0 g
Potassium phosphate (dibasic)	0.5 g
L-Cysteine (monohydrochloride)	1.0 g
Ferric ammonium citrate	22.0 mg
Ascorbic acid	200.0 mg

Distilled water	875.0 ml

pH was adjusted to 6.8 by 1N NaOH

The broth was suction filtered in Buchner funnel through Whatman No.1 filter paper, autoclaved at 15 lbs pressure (121°C) for 15 min and allowed to cool to room temperature.

Vitamin Tween-80 mixture (For TYI-S-33 medium)

Solution A

Vitamin mixture No.107 (prepared as described).

Solution B

Vitamin B_{12} (Sigma Chem. Co.) : 40.0 mg dissolved in distilled water and final volume made to 100 ml.

Solution C

DL-6,8-thioctic acid (Sigma Chem. Co.) : 100.0 mg dissolved in 100 ml absolute ethyl alcohol.

Solution D

Tween-80 (Sigma) : 50.0 g Tween-80 was dissolved in 100 ml of absolute ethyl alcohol.

The final mixture was constituted as follows :

Solution A	100.0 ml
Solution B	12.0 ml
Solution C	4.0 ml
Solution D	4.0 ml

Final volume was made to 180.0 ml with distilled water. This working solution was sterilized by passing through 0.2 μM poly-

carbonate filter (Millipore) and stored at -20°C in the dark in brown bottles.

Constitution of the complete TYI-S-33 medium

The constituents of complete TYI-S-33 medium for 100 ml are :

Autoclaved TYI-S-33 nutrient broth	87.0 ml
Sterilized serum-vitamin solution (80:20)	13.0 ml
Penicillin	0.25 ml
Streptomycin	0.25ml
Terramycin	0.25ml

Aseptic conditions were maintained throughout the preparation of medium.

CHOLESTEROL TREATMENT

The procedure of Bos and Van de Griend (1977) was used to enrich the medium with cholesterol. Thirty five milligrams of extra fine cholesterol was dissolved in 10.0 ml of chloroform and 100 µl of this solution (350 µg cholesterol) was pipetted into 125x15 mm screw capped tubes and made to coat as much of the inner surface as possible. With screw caps loosened, the chloroform was allowed to evaporate at room temperature. The tubes, after being autoclaved at 15 lbs pressure (121°C) for 15 min, were allowed to cool and dry at room temperature and were finally filled up with 12.0 ml of the appropriate sterile medium.

CULTURING PROCEDURE

The complete medium was dispensed in screw capped tubes (125x15 mm) to check the sterility of this medium. The tubes

were incubated at 37°C for 24-48 hrs before use. These tubes were inoculated with 10,000 to 20,000 amoebae from a 72 hrs old parent culture and incubated at 37°C in the upright position. Subculturing was carried out at every 72 hr.

Clonal culture

The clonal cultures were established by the procedure of Das and Meerovitch (1981) by growing single cell of the amoeba in TYI-S-33 medium in a specially designed sealed 'perspex chambers' from the parent culture (DKB strain). When a fairly good number of trophozoites had developed from the isolated single amoeba, the contents of the cavity were transferred into screw capped culture tubes containing fresh medium and the culture tubes were maintained as described.

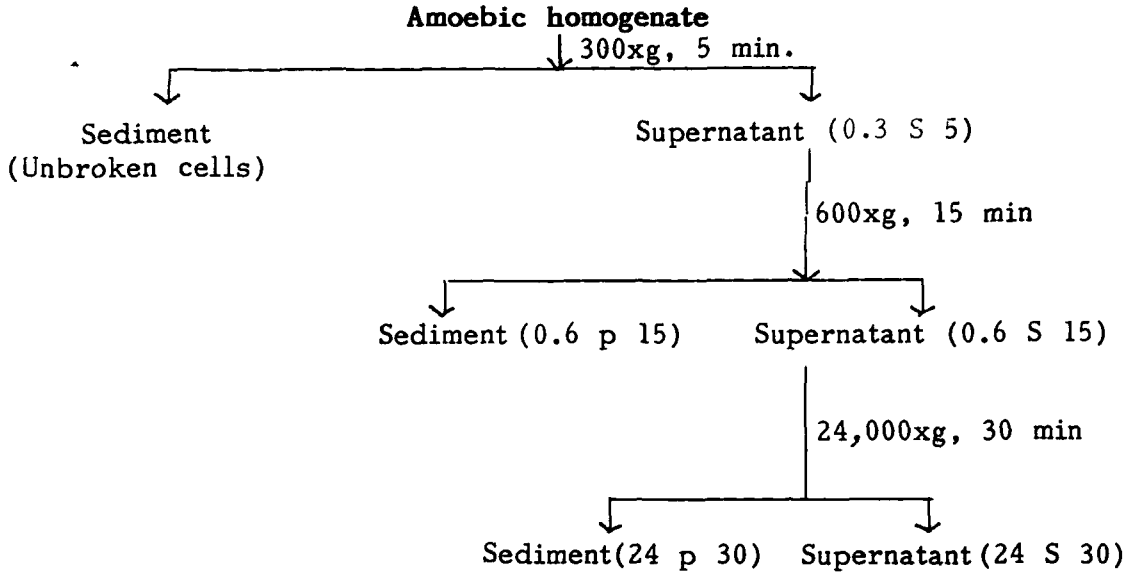
HARVESTING OF AMOEBAE

Cultures, during their log phase of growth (72 hr, at 37°C, unless specified otherwise) were chilled in ice cold water for 5 min to dislodge the amoebae from glass surface. The culture tubes were than centrifuged at 300xg for 5 min followed by at least three washings (for complete removal of the medium constituents) with 0.85% sodium chloride (normal saline). The supernatant was decanted and the amoebae in the pellet were resuspended in phosphate buffer saline, pH 7.2 (unless mentioned otherwise). The cell number of final amoebic suspension was counted in a haemocytometer.

SUB-CELLULAR FRACTIONATION

The procedure employed for fractionation of amoebic cells was similar to that of Hogeboom (1955). Various fractions obtained have been designated according to the nomenclature adopted by Alexander (1956).

Details of the fractionation procedure is given in the flow diagram :



CONCANAVALIN A AGGLUTINATION ASSAY

Con A agglutination was done according to Prasad et al. (1981). This was assayed in terms of microscopic aggregation index (MAI) determined through haemocytometer counting of cells and their aggregates. Equal volumes of lectin solution (0.05 ml of 30.0 µg/ml solution) and amoebic suspension were mixed together directly on the haemocytometer and were allowed to interact at room temperature (approximately 25°C) for 5 min. A control sample of the same amoebic suspension mixed with an equal volume of

phosphate buffered saline (PBS) and similarly counted in all the experiments. The MAI were determined according to the method of Chien (1975) (developed to estimate erythrocytic aggregation), which was defined as

$$MAI = \frac{\text{Number of units in control}}{\text{Number of units in presence of lectin}}$$

HAEMOLYTIC ASSAY

Haemolytic assay procedure was essentially same as described by Prasad et al. (1982). The **E.histolytica** cell suspension (10^6 cells/ml) in phosphate buffered saline (PBS), pH 7.2, were homogenised by 25-30 strokes in a Potter-Elvehjam homogeniser. Large cells debris and residul intact cells were removed by low speed centrifugation. The sediment was resuspended in a small volume of PBS, and was similarly re-homogenised and centrifuged to finally pool the two supernatants. The homogenate thus prepared showed almost complete absence of intact amoebae on microscopic examination.

Erythrocyte haematocrit (20%) in PBS was incubated overnight (16-18 hr) with an equal volume of appropriately diluted homogenate so that the amoebal protein content in the assay mixture was equal to 250 μg/ml. The degree of haemolysis was finally estimated in terms of spectrophotometric measurement of haemoglobin released in the centrifuged supernatant at 440 nm. This was expressed as percent of the total haemoglobin released by osmotic lysis with distilled water in an equivalent quantity of the erythrocytes. A similarly incubated control in which amoebic homogenate was replaced by euqal volume of PBS was invariably

set up and respective haemolysis value was substracted to compute the presented relative haemolysis data.

ERYTHROPHAGOCYTOSIS ASSAY

Erythrophagocytosis by different cultures was assayed using a modification of the procedure employed by Trissl et al. (1978). A suspension of erythrocytes for these studies was prepared from blood drawn from the eye of the rats through a sharp capillary. The erythrocytes were separated and washed three times with the physiological saline rejecting the top buffy coat. The density of RBC was adjusted finally to 10^8 cells/ml in saline by haemocytometer counting.

For erythrophagocytosis assay, 0.2 ml of the amoebic cell suspensions (10^6 cells/ml) in the appropriate growth medium were incubated with 0.2 ml of above RBC preparation for the desired time at room temperature (28-30°C). Ten ml of distilled water was then added rapidly for eliminating the external RBC attached to amoebic surface. After centrifugation at 600xg for 1 min, the pellet was re-suspended and fixed for 15 min in glutaraldehyde (0.2% in phosphate buffer saline, pH 7.0). The cells thus fixed were washed with PBS and phagocytosed erythrocytes in at least 100 amoebae were counted in a haemocytometer. The degree of phagocytosis was expressed in terms of average number of RBC per amoebic cell.

EFFECT OF SUGARS ON ERYTHROPHAGOCYTOSIS

To study the effect of different sugars on erythrophagocytosis, cell suspension of amoebae and RBCs were pre-incubated at room temperature (28-30°C) separately with 50 mM of sugars for

10 min. The two were then mixed together and the erythrophagocytosis was assayed microscopically in terms of average number of engulfed erythrocytes per amoeba.

PREPARATION OF ^{14}C-SUCROSE LOADED RBC GHOSTS

The ^{14}C-sucrose loaded RBC ghosts were prepared according to the procedure of Bodemann and Passan (1972). Blood was collected from the eye of rats using a sharp capillary under sterile conditions in heparin (anticoagulant). After centrifugation at 1,600xg for 10 min the buffy coat was carefully removed and ghosts were prepared as summarized below :

Step I - RBC were washed three times in isotonic sodium chloride.

Step II- Preparation of 50% cell suspension (v/v) in isotonic tris solution, pH 7.4.

Step III- Hemolysis of 1.0 ml of 50% RBC suspension by 10.0 ml of 10 mM Tris buffer containing 4 mmole/L of $MgSO_4$ (hypotonic hemolysing medium) at 0°C for 5 min. This buffer contains 5 µCi of ^{14}C-(U)Sucrose (specific activity (580 mCi/mmol.) and 1 mM unlabelled sucrose.

Step IV -Restoration of isotonicity by 0.5 ml of 1.8 M KCl and kept for 50 min at 37°C (for resealing of RBC ghosts).

Step V - Centrifuge at 24,000xg for 10 min and then washed with isotonic tris-NaCl buffer of pH 7.4

Phagocytosis of ^{14}C-sucrose loaded erythrocyte ghosts

For RBC ghost phagocytosis assay, 0.2 ml of the amoebic cell suspension (10^6/ml, normal saline) was incubated with 0.2 ml

of ^{14}C sucrose loaded RBC ghost (approximately 10^8/ml) for desired time at room temperature. Then 10.0 ml of distilled suspension water was added rapidly to lyse unphagocytosed ghosts. After centrifugation at 600xg for 1 min, the pellet was washed two times in normal saline and added to scintillation fluid for radioactivity measurement in LKB Beeta Liquid Scintillation Counter.

For studying the effect of different sugars on the ghost phagocytosis, the amoebic cell suspension was preincubated with 50 mM sugar solution at room temperature for 10 min and then phagocytosis was followed as above.

MEASUREMENT OF GLUCOSE METABOLISM ($^{14}CO_2$ production)

Measurement of $^{14}CO_2$ production from ^{14}C-(U)-glucose was carried out according to the method of Kelman et al. (1981), using Warburg flasks. Two ml suspension of erythrocytes (10^8 Cells), **E.histolytica** (10^6 cells) or both in Kreb's Ringer phosphate buffer was taken in flask and the reaction was started by the addition of 5 mmoles/L glucose supplemented with 0.25 μCi ^{14}C-(U)-glucose (specific activity 160 mCi/mmol.). The central well of these flasks contained filter paper soaked with 0.2 ml of 2M KOH, which were after being highly stoppered incubated in a shaking water bath for 1 hr at 37°C. The reaction was stopped by adding 0.7 ml of 35% Perchloric acid and the flasks further incubated for 30 min to ensure that released $^{14}CO_2$ was fully trapped in the KOH soaked filter papers. Appropriate control in which perchloric acid was added before the addition of glucose was always simultaneously set up which gave values of less than 10% of the experimental samples. Contents of the well (KOH with trapped

$^{14}CO_2$) were transferred in scintillation fluid and counted in Liquid Scintillation Counter (LKB).

GLUCOSE OXIDATION ASSAYS

Glucose oxidation was followed in amoebic and amoebic erythrocyte suspension. Erythrocyte suspension for these studies was prepared from blood drawn from the eye of the rats through a sharp capillary. After centrifugation, plasma and white cells were removed, and red cells were washed three times with phosphate-buffered saline, pH 7.4. A 25% (v/v) suspension of red cells was prepared in Krebs-Ringer phosphate buffer (100 parts, 0.154 M NaCl; 4 parts, 0.154 M KCl; 1.5 parts, 0.11 M $CaCl_2$; 1 part, 0.154 M $MgSO_4$; 21 parts, 0.1 M NaH_2PO_4/NaH_2PO_4, pH 7.4) without glucose.

E.histolytica culture suspension (10^6 cells/ml) was prepared by suspending washed cells (as decribed earlier) in above Krebs Ringer phosphate buffer.

Measurement of oxygen uptake ($E\text{-}QO_2$)

The rate of endogenous oxygen uptake ($E\text{-}QO_2$) by **E.histolytica/bacteria/RBC** was measured polarographically in closed system of Gilson Oxygraph Model 5/6H using the procedure employed by Mendis and Townson. (1985). **E.histolytica/RBC/bacteria** or mixture of two cells were introduced into assay chamber containing 1.8 ml air saturated Krebs Ringer bicarbonate buffer, pH 7.4 and equilibrated with contant stirring for 5 min at 37°C. The electrode chamber was then closed and uptake rate assayed. The oxygen consumption by the electrode *per se* was monitored prior to each

assay without sample. EQO_2 value of sample was corrected for this factor.

NBT REDUCTION ASSAY

Amoebic cells harvested from 72 h old cultures were suspended to a density of $3.3x10^6$ cells/ml in Hank's buffer (without glucose). They were homogenised in a Potter Elvehjam homogenizer to disrupt more than 99% cells, as confirmed by haemocytometer counting. The homogenate were differentially centrifuged to isolate its particulate components sedimentable at 0.6 p 15 and 24 p 30 and the supernatant 24 S 30. The nomenclature of Alexander (1956) was used as described earlier to designate the fractions.

Estimation of NBT reduction was carried out using the procedure adopted by Aust-Kettis et al. (1982). Reaction mixtures containing cell-suspensions, homogenate or isolated fractions with 0.05% NBT (in a total volume of 1.0 ml Hank's buffer) were incubated in a shaking water bath at 37°C for 1 hr (unless mentioned otherwise). The reaction was interrupted at this stage by the addition of 1.0 ml 0.5 N HCl and the samples were centrifuged at 24,000xg for 10 min. The formazan associated with the pellets was extracted with 1.5 ml Dimethyl sulphoxide (DMSO) and the optical density (OD) of the extract was read at 572 nm (against the respective zero time control), which was directly taken as the index of relative NBT reduction.

RADIO-IODINATION OF E.HISTOLYTICA SURFACE PROTEIN BY IODOGEN METHOD

The iodination of **E.histolytica** surface proteins was done according to the method of Howard et al. (1982). The iodinating

reagent 1,3,4,6 tetrachloro 3 α, 6 α, diphenyl glycoluril (IODOGEN) was used to label **E.histolytica** surface proteins.

Cylindrical capped glass vials (45x15 mm) were coated with IODOGEN (Pierce Chemical Co. Rockford, Illinois) dissolved in dichloromethane (0.1 mg/ml) using a stream of nitrogen gas to evaporate the solvent (625 µl IODOGEN solution). The tubes were gently shaken during evaporation in order to coat the bottom of the tube evenly with IODOGEN. Immediately before the use, tubes were gently rinsed twice with phosphate buffered saline (PBS), pH 7.4. For radiolabelling $3.3x10^6$ amoebic cells/ml PBS were added to lthe IODOGEN coated tubes and reaction was initiated by additing 0.5 mCi of $Na^{125}I$ (5 µl of 5 mCi/50 µl solution). After incubation for upto 5 min at 28^oC with continuous gentle shaking, the cell suspension was added into 15.0 ml of ice cold PBS containing 5 mM NaI to terminate the reaction. The cells were washed twice in PBS containing NaI and thrice in PBS.

ALCOHOL DEHYDROGENASE ASSAY

Alcohol dehydrogenase activities in both backward (NADH/ NADPH oxidation by acetaldehyde) and forward directions (reduction of these co-enzymes by alcohol) were assayed in terms of absorbance changes at 340 nm using **E.histolytica** extracts of 35,000xg for 30 min (35 S 30) as the enzyme source, employing a slightly modified procedure of LO and Reeves (1978).

In case of backward direction, the reaction mixture in a total volume of 3.0 ml consisted, potassium phosphate buffer, pH 6.5, 20 µmoles; acetaldehyde, 23 µmole; NADH or NADPH, 0.2 µ moles and a suitable amount of enzyme proteins. The rate of oxida-

tion of NADH/NADPH was measured by recording decrease in absorbance at 340 nm in a Shimadzu double beam UV-190 spectrophotometer for 5 min. One unit of the enzyme was defined as the amount causing the oxidation of 1 nmole of NADH/NADPH per min under our experimental conditions and specific activity was expressed as units $(mg\ protein)^{-1}$.

Enzyme activity was also assayed in the forward direction i.e. ethanol to acetaldehyde in 50 mM glycine-NaOH buffer, pH 9.5 containing 0.2 mM NADP/NAD and 0.16 M ethanol in terms of increase in absorbance at 340 nm. One unit of enzyme was defined as the amount causing the production of 1 nmole of NADH/NADPH per min under our experimental conditions.

To study the effect of inhibitors/activators, the enzyme source was preincubated for 10 min at 37°C and then reaction was followed as described above.

ACID PHOSPHATASE ASSAY

The **E.histolytica** cells were harvested (as described earlier) and homogenized in Potter-Elvehjem homogenizer at 4°C with prechilled 0.25 M sucrose solution. The resulting homogenate was centrifuged at 600xg for 15 min and supernatant was used for enzyme assay.

Acid phosphatase in **E.histolytica** homogenate/extract was assayed according to Somer (1954) with minor modification using p-nitrophenyl phosphate as substrate. The assay mixture contained 6 µM of substrate; 0.1 M acetate buffer pH 4.5; 3 µM EDTA and 0.02 ml of the sample in total volume of 2.0 ml. In some cases

triton X-100 (final concentration 0.1%) was added to the assay mixture. Samples were incubated at 37°C for desired time (usually 30 min), the colour was developed by adding 2.0 ml of 0·1 N NaOH to the reaction mixture and O.D. was read at 410 nm in Shimadzu double beam spectrophotometer. One unit of enzyme represented the quantity which produced 1 n mole p-nitrophenol per min under the described assay conditions.

PROTEINASE ASSAY

Washed **E.histolytica** cells (as described earlier) were homogenized in normal saline containing 2 mM DTT and centrifuged at 10,000xg, 20 min, 4°C. Sediment was treated with 0.5% triton X-100 in above solution and resulting extract was used as an enzyme source.

Proteinase activity was followed according to the method of McLaughlin and Faubert (1977), using 0.2 ml of 1% azocasein in 0.2 M sodium phosphate buffer, pH 6.0, with enzyme added to give a final volume of 0.3 ml and incubated at 37°C for 1 hr. Subsequently 0.5 ml of 0.5 M trichloroacetic acid was added to each tube and were allowed to stand for 10 min, centrifuged for 15 min at 10,000xg, after which 2.0 ml of 0.5 N NaOH was added to each supernatant and the absorbance was read at 420 nm in Shimadzu double beam spectrophotometer. Unit of enzyme activity was expressed as a change of absorbance of 0.10/hr and specific activity was expressed as units/mg protein.

For the study of inhibition/activation the enzyme source was pre-incubated with desired amount of inhibitor/activator at 37°C for 10 min and then reaction was carried out as above.

ELECTROPHORETIC SEPARATION OF ACID PHOSPHATASE ISOENZYMES

Polyacrylamide disc gel electrophoresis of amoebic extract was conducted according to the method of Davis (1964). Stock solutions of the following were made for the preparation of polyacrylamide gels :

A. Acrylamide (Sigma) 28% + Bisacrylamide (Sigma) 0.75% (w/v).

B. Electrophoretic buffer : glycine (Sigma) 0.29% + Tris 0.06% (w/v) pH 8.3.

C. N'-N'-N'-tetramethyl ethylene diamine (TEMED) (Sigma) 8.28% (v/v)

D. Ammonium per sulphate (Sigma) 0.14% (w/v).

E. Gel buffer, Tris 36.6% (W/v) pH 8.9.

A mixture of the above A,B,C,D was prepared in the ratio of 2:1:1:4 distributed in gel tubes and kept in the dark for 45 min for solidification and polymerisation.

Sufficient quantity of electrophoretic buffer was filled in electrophoretic chamber after placing the polyacrylamide gel tubes. Each sample containing 75-100 µg of protein in 0.01 ml (containing small amount of marker dye) was applied to the polyacrylamide gel. A current of 5 mA per gel tube at 200 volts was supplied using a electrophoretic power pack. The gel tubes were removed as marker dye reached the opposite end of the tube. The gels were taken out with the help of a syringe. After electrophoresis the gels were washed with respective buffers used for detection of enzymes on gels.

Acid phosphatase isoenzyme were detected by using acetate buffer, (0.1 M, pH 5.0), sodium 2-napthyl phosphate and Garnet GBC as colour developer by the method of Podhajcer et al. (1985).

Movement of bromophenol blue on the gel was simultaneously followed and the ratio of the distance travelled by the enzyme band and the dye (relative mobility:Rm) was determined.

PYRUVATE PHOSPHATE DIKINASE (PPD) ASSAY

PPD was assayed according to the procedure of Milner et al. (1975). Washed **E.histolytica** cells were suspended in three volumes of cold 0.05 M potassium phosphate buffer, pH 7.0 containing 0.25 M sucrose and 1 mM dithiothreitol and ruptured by 20 strokes in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 35,000xg for 30 min and the supernatant was used for the enzyme assay. Properly diluted enzyme in 0.05 ml was added to the test tubes and then 0.1 ml each of pyrophosphate solution (30 mM, pH 6.7) and 0.05 ml of adenosine monophosphate (60 mM, pH 6.7) was added. The reaction was initiated by adding 0.3 ml of the substrate mixture (1 ml mixture containing imidazole HCl buffer, pH 6.7, 80 µmoles; $(NH_4)_2SO_4$, 40 µmoles; p-enolpyruvate, 4.8 µmoles and $MgCl_2$ 24 µmoles). The tubes were incubated 25°C for 30 min. The controls in which AMP was replaced by an equivalent of imidazole-HCl buffer ran similarly in each experiment. The reaction was terminated by adding 0.3 ml of dinitrophenyl hydrazine reagent (0.1% in 2 N HCl). After 5 min, 0.2 ml of 2 N HCl solution was added and the absorbance was read after 10 min in a spectrophotometer at 415 nm. For calculating the enzyme activity the value of molar extention coefficient was taken as ϵ 415 = 1.1 x 10^7 $mole^{-1}$ lit^{-1} for the hydrazone formed during the reaction. The enzyme unit was expressed as amount of pyruvate formed/min and specific activities was expressed as unit/mg protein.

PYRUVATE KINASE ASSAY

Pyruvate kinase of rat liver supernatant (10% homogenate, 10,000xg, 20 min) was assayed by slight modified method of Bucher and Pfleiderer (1955) using following protocol. The assay mixture contained phosphate buffer, pH 7.5, 25 mM; ADP, 0.0115 mM; phosphoenolpyruvate, 0.036 mM; $MgSO_4$, 0.4 mM; KCl, 3.75 mM and suitable amount of enzyme protein in total volume of 2.0 ml. The reaction was terminated after 30 min incubation at 37°C by 2.0 ml 10% TCA. The precipitate was centrifuged and the pyruvate formed was estimated in terms of phenyl hydrazone formation by dinitrophenyl hydrazine at 415 nm.

PROTEIN ESTIMATION

The protein content of the samples were estimated calorimetrically using a slight modification of Lowry's method (Lowry et al., 1951). Eight percent (w/v) solution of anhydrous sodium carbonate was mixed with equal volume of solution containing 0.06% (w/v) of cupric sulphate and 0.12% (w/v) sodium potassium tartarate. Solutions containing 10 to 150 µg of protein were mixed with 5.0 ml of the above reagent, incubated for 10 min at 37°C and then cooled to room temperature. 0.5 ml of the twice filuted Folin phenol reagent was added and the colour developed was read at 660 nm after 30 min against a proper blank in spectrophotometer using bovine serum albumin as standard. Under these conditions optical density equivalent to one Klett division was found to be equal to one microgram of standard protein.

CHAPTER III

RESULTS

A. VIRULENCE ASSAY AND PAHGOCYTOTIC STUDIES OF E.HISTOLYTICA

VIRULENCE ASSAY

The virulence of the cultures used was checked occasionally in terms of lesion-forming ability in golden hamsters (Mesocricetus auratus) liver by inoculating 50,000 amoebae using the procedure of Dutta (1970). This method reproducibly induced pus-containing lesions with IP:106 strain (4 grade, 100%) and cholesterol passaged NIH:200 strain (3 grade, 100%) in terms of a semiquantitative morphology based grading procedure fell under the +++ or ++++ category. On the other hand normal unpassaged NIH:200 and DKB strains did not induce any lesions under similar conditions or only occasional 'pin head' lesions in case of NIH:200 (2 grade, 50%) (Table 1, Fig. 1-4).

Concanavalin A (Con A) agglutination and haemolytic activity were measured in the culture as in vitro indices of their virulence. Both the activities were found to be consistently maximum in IP:106 strain and minimum in the DKB strain. Further, these activities increased significantly after cholesterol passaged of NIH:200 strain (Table 2).

ERYTHROPHAGOCYTOSIS

Effect of medium/buffers on erythrophagocytosis

With a view to examine whether exogenous organic nutrients influence the rate of erythrophagocytosis of **E.histolytica**, it was followed in different media/buffers. The results (which are presented in Table 3) showed almost the same rate of erythrophagocytosis in all the media/buffers used suggesting that exogenous substrates do

not influence erythrophagocytotic ability of **E.histolytica** under our experimental conditions.

Effect of temperature

The rate of erythrophagocytosis at different temperatures are presented in Table 4. These results showed that the rate of phagocytosis at 25°C and 37°C is nearly same, while there is no phagocytosis at 4°C, although the RBCs were found to adhere to amoebae even at this temperature.

Comparison in different E.histolytica strains and their clones

Comparative results on progress of erythrophagocytosis in three different strains of **E.histolytica** are presented in Fig. 5. The data showed very marked difference in the erythrophagocytotic capacity of IP:106 and DKB cultures, whereas this activity of NIH:200 strain fell in between the two. Statistically significant differences were, however, indicated in the clonal cultures derived from the DKB strain which showed lowest erythrophagocytotic capacity amongst the above three strains (Fig. 6). The highest erythrophagocytotic activity, amongst the clones was noted in DKB-3 clone which was found earlier to possess higher specific activities of several other biochemical indicators of virulence (Katiyar et al., 1989).

Effect of cholesterol passage of E.histolytica culture

Serial passage of NIH:200 culture through cholesterol-enriched modified Diamond's TPS-II medium (a strain whose virulence in animals has been shown earlier to markedly increase by the sterol treatment;

(Das and Ghoshal, 1976) resulted in considerable increase in the erythrophagocytotic capacity of the culture (Fig. 7).

Effect of sugars

Effect of different sugars on the erythrophagocytotic capacity of NIH:200 and IP:106 cultures are presented in Table 5. The data show considerable reduction in the degree of erythrophagocytosis in both these cultures by N-acetyl-galactosamine, galactose and lactose at all the time points and slight but statistically significant decrease by fructose. The other sugars tested showed only marginal influence on this activity, if any, in either strain.

PHAGOCYTOSIS OF ^{14}C-SUCROSE LOADED RBC GHOSTS

Resealed erythrocyte ghosts packed with ^{14}C-sucrose were prepared and their phagocytosis was followed in terms of uptake of radioactivity in the trophozoites as described under 'Materials and Methods'. Comparative results on phagocytosis of such ghosts in three **E.histolytica** strains are presented in Fig. 8. These results show that there are marked differences in relative radioactivity in different strains after the different time incubation with RBC ghosts. The DKB strain once again showed lowest radioactivity uptake and IP:106 strain showed the highest while NIH:200 falls in between the two. Further, cholesterol passage of NIH:200 culture also markedly increased the ghost phagocytosis (Fig. 9), however, pre-treatment of amoebae and RBC ghosts with 50 mM N-acetyl-D-galactosamine significantly decreased the rate of phagocytosis in all the three strain

(Fig. 10, 11, 12), indicating that the cytosolic constituents of RBC are not involved in the mechanism of inhibition of erythrophagocytosis by this sugar.

INTERNALIZATION OF ^{125}I-LABELLED CONCANAVALIN A

Concanavalin A (Con A) was labelled, using 0.5 mCi Na ^{125}I (5 µl of 1 mCi /10 µl solution) and 500 µg Con A in 1 ml of phosphate buffered saline, pH 7.4, which were made to interact in IODOGEN coated tube according to the method of Harward et al. (1982) and labelled Con A separated from free ^{125}I by using Sephadex G-50 column chromatography. **E.histolytica** cells (10^6 cells) in 1.0 ml of Hank's buffer, pH 7.2 (with or without α-methyl mannoside, 50 mM, or bovine serum albumin 0.1%) were incubated with 50 µg ^{125}I-labelled Con A (approx. 50,000 cpm) for desired intervals at 30°C. These lectin exposed cells were then sedimented (600xg, 5 min) and washed twice with 0.5M α-methyl mannoside containing Hank's buffer to remove external lectin bound with **E.histolytica** surface and residual radioactivity associated with the cells was taken as an index of internalization of the lectin.

Maximum internalization of Con A was observed in IP:106 strain, amongst the three cultures tested, at all the time points and NIH:200 strain grown in cholesterol enriched medium gave higher internalization than normal NIH:200 (Table 6). The pre-treatment of **E.histolytica** cells with α-methyl mannoside (which is specific sugar hapten for Con A) markedly decreased internalization of lectin by apparently inhibiting interaction of Con A with its receptors on **E.histolytica**

surface. However, pre-treatment of these cells by bovine serum albumin, on the other hand, increased somewhat internalization of the lectin in all the three strains (Table 6).

$^{14}CO_2$ PRODUCTION IN DIFFERENT E.HISTOLYTICA STRAINS DURING RBC ADHERENCE/PHAGOCYTOSIS

Relative quantities of $^{14}CO_2$ generated from ^{14}C-(U)-glucose by **E.histolytica** cultures alone and in presence of RBC are presented in Table 7. There was markedly higher CO_2 production (per amoebic cell) by IP:106 strain of **E.histolytica** as compared to NIH:200 strain and this increased significantly by growing the latter in cholesterol enriched medium. Further, this activity in a mixture of amoeba and RBC was higher compared to sum of CO_2 production by equivalent amount of amoebae and RBC alone. This stimulation of $^{14}CO_2$ generation by RBC was maximum in IP:106 (128.8%) followed by cholesterol passaged NIH:200 (59.9%) and NIH:200 (34.32%).

B. OXIDO-REDUCTIVE FUNCTIONS OF E.HISTOLYTICA

NITROBLUE TETRAZOLIUM (NBT) REDUCTION STUDIES

Aust-Kettis et al. (1982) followed the reduction of NBT by **E.histolytica** cells in Eagle's minimal medium. Experiments were undertaken to investigate whether the organic nutrients present in this medium, or other substrates known to enhance its respiratory activity (O_2uptake, Weinbach and Diamond, 1974) support NBT reduction. The results (Table 8) showed more or less identical dye reduction

Table 1: Pathogenicity of different **E.histolytica** strains against experimental hepatic amoebiasis of golden hamsters (Mesocricetus auratus).

Strains of **E.histolytica**	No. of trophozoites inoculated	No. of hamsters inoculated/ infected	No.of hamsters showing lesions	Percent infected hamsters	Average hepatic score (grade of lesion) Maximum=4
DKB	50,000	12/0	NIL	NIL	1.0
NIH:200	50,000	12/6	6	50	2.0
NIH:200 (Cholesterol passaged)	50,000	12/12	12	100	3.0
IP:106	50,000	12/12	12	100	4.0

Table 2: Con A agglutinability and haemolytic potency of **E.histolytica** cultures.

Cultures	Relative haemolysis[+] (%)	Con A agglutinability* (MAI values)
DKB	1.7±0.74	1.20±0.10
NIH:200	22.0±3.60	1.80±0.19
NIH:200 (Cholesterol passaged)	50.0±5.80	3.40±0.38
IP:106	49.5±5.80	5.20±0.32

Data are mean±S.D. of three independent experiments

*Con A concentration was 15.0 µg/ml

+Haemolytic activity assayed using 250 µg/ml homogenate protein.

Table 3: Rate of erythrophagocytosis in NIH:200 strain of **E.histolytica** in different medium/buffers.

Medium/Buffer	Erythro phagocytosis (RBC/amoeba)	
	20 min	40 min
Diamond's TPS-II medium	3.42±0.23	4.51±0.36
Eagle's medium	3.39±0.33	4.57±0.39
Hank's buffer	3.06±0.24	4.32±0.30
Kreb's Ringer buffer	3.10±0.16	3.96±0.27
Normal saline	2.96±0.27	3.76±0.32

Values are mean±S.D. based on data obtained in three independent experiments.

Table 4: Effect of temperature on erythrophagocytosis of **E.histolytica** (NIH:200).

Temperature (^{o}C)	RBC/amoeba after 30 min incubation
0	0.00
4	0.00
25	3.87
37	3.92

All values are mean of three independent sets of experiments.

Table 5: Effect of various sugar molecules on the erythrophagocytotic ability of **E.histolytica** cultures.

Sugars	NIH:200			IP:106		
	10 min	20 min	40 min	10 min	20 min	40 min
Control	1.49±0.144	3.38±0.191	4.35±0.372	3.63±0.035	6.13±0.231	8.35±0.480
N-acetyl-D-galactosamine	0.392±0.015	0.903±0.097	1.24±0.040	0.93±0.062	2.41±0.168	4.50±0.245
	P<0.005	P<0.001	P<0.001	P<0.001	P<0.001	P<0.05
Galactose	0.436±0.029	0.905±0.047	1.30±0.110	1.06±0.079	2.89±0.259	5.07±0.185
	P<0.005	P<0.001	P<0.005	P<0.001	P<0.001	P<0.001
Lactose	0.639±0.089	1.24±0.200	2.31±0.290	1.87±0.110	3.23±0.150	6.07±0.136
	P<0.005	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001
Fructose	1.14±0.112	2.51±0.153	3.54±0.056	2.22±0.160	3.90±0.100	6.05±0.110
	P<0.05	P<0.01	P<0.05	P<0.001	P<0.001	P<0.001
Glucose	1.33±0.052	3.32±0.185	4.14±0.158	3.27±0.187	3.93±0.066	8.24±0.066
	NS	NS	NS	NS	NS	NS
Rhamnose	1.35±0.035	2.93±0.066	4.10±0.060	3.40±0.085	5.74±0.140	7.90±0.080
	NS	NS	NS	NS	NS	NS
N-acetyl glucosamine	1.41±0.020	3.39±0.070	4.42±0.260	3.41±0.215	6.15±0.047	8.25±0.077
	NS	NS	NS	NS	NS	NS

Data are mean±SD of three experiments. P-values relate to difference from respective control data.
NS - Difference statistically not significant (P>0.05).

Table 6: Concanavalin A internalization in different strains of **E.histolytica**.

Strains	Incubation time (min)	Concanavalin A internalization (CPM/10^6 cells of **E.histolytica**)		
		No treatment	Cells pre-treated with α-methyl-mannoside	Cells pre-treated with bovine serum albumin
NIH:200	0	1,052	595	1,457
	30	3,295	1,266	5,776
	60	5,610	2,670	8,510
NIH:200 (Cholesterol passaged)	0	1,156	676	1,369
	30	5,672	2,695	8,872
	60	9,575	4,676	12,764
IP:106	0	1,095	644	1,372
	30	8,566	3,596	12,800
	60	12,601	5,777	15,995

Table 7: $^{14}CO_2$ Production in **E.histolytica** cultures alone and in presence of RBC.

Strains	$^{14}CO_2$ production		% Increase in CO_2 production
	n moles/hr/10^6 **E.histolytica** cells	nmoles/hr/10^6 **E.histolytica**+10^8RBC*	
NIH:200	4.96×10^{-3} (100)	10.98×10^{-3}	34.32
NIH:200 (Cholesterol passaged)	8.30×10^{-3} (167)	17.26×10^{-3}	59.90
IP:106	10.63×10^{-3} (214)	28.38×10^{-3}	128.78

*$^{14}CO_2$ produced per 10^8 RBC = 4.061×10^{-3} nmoles/hr

Values in parenthesis represent the relative CO_2 production in different strains. Data are average of three independent experiments.

Fig.1. Hamster liver showing, 1 grade liver lesion *in situ* after the inoculation of 50,000 trophozoites of DKB strain of ***E.histolytica*** (xenic).

Fig.2. Hamster liver showing, 2 grade liver lesion *in situ* after the inoculation of 50,000 trophozoites of NIH:200 strain of ***E.histolytica*** (axenic).

Fig.3. Hamster liver showing, 3 grade liver lesion *in situ* after the inoculation of 50,000 trophozoites of cholesterol passaged NIH:200 strain of ***E.histolytica*** (axenic).

Fig.4. Hamster liver showing, 4 grade liver lesion *in situ* after the inoculation of 50,000 trophozoites of IP:106 strain of ***E.histolytica*** (axenic).

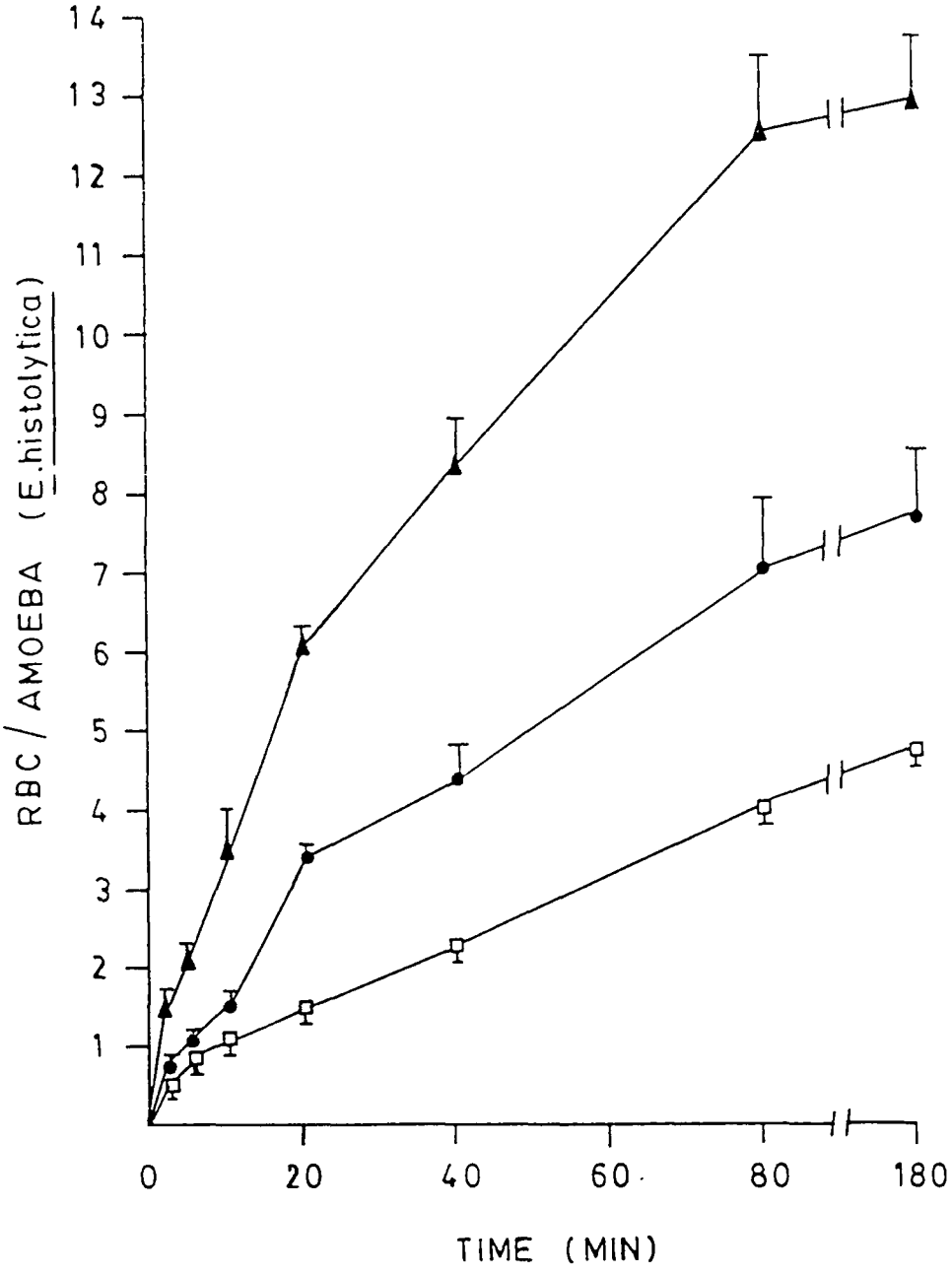


Fig.5. Comparison of the progress of RBC phagocytosis with time in IP:106 (▲-▲), NIH:200 (●-●) and DKB (□-□) strains of **E.histolytica**. The bars represent SD values obtained from three independent experiments.

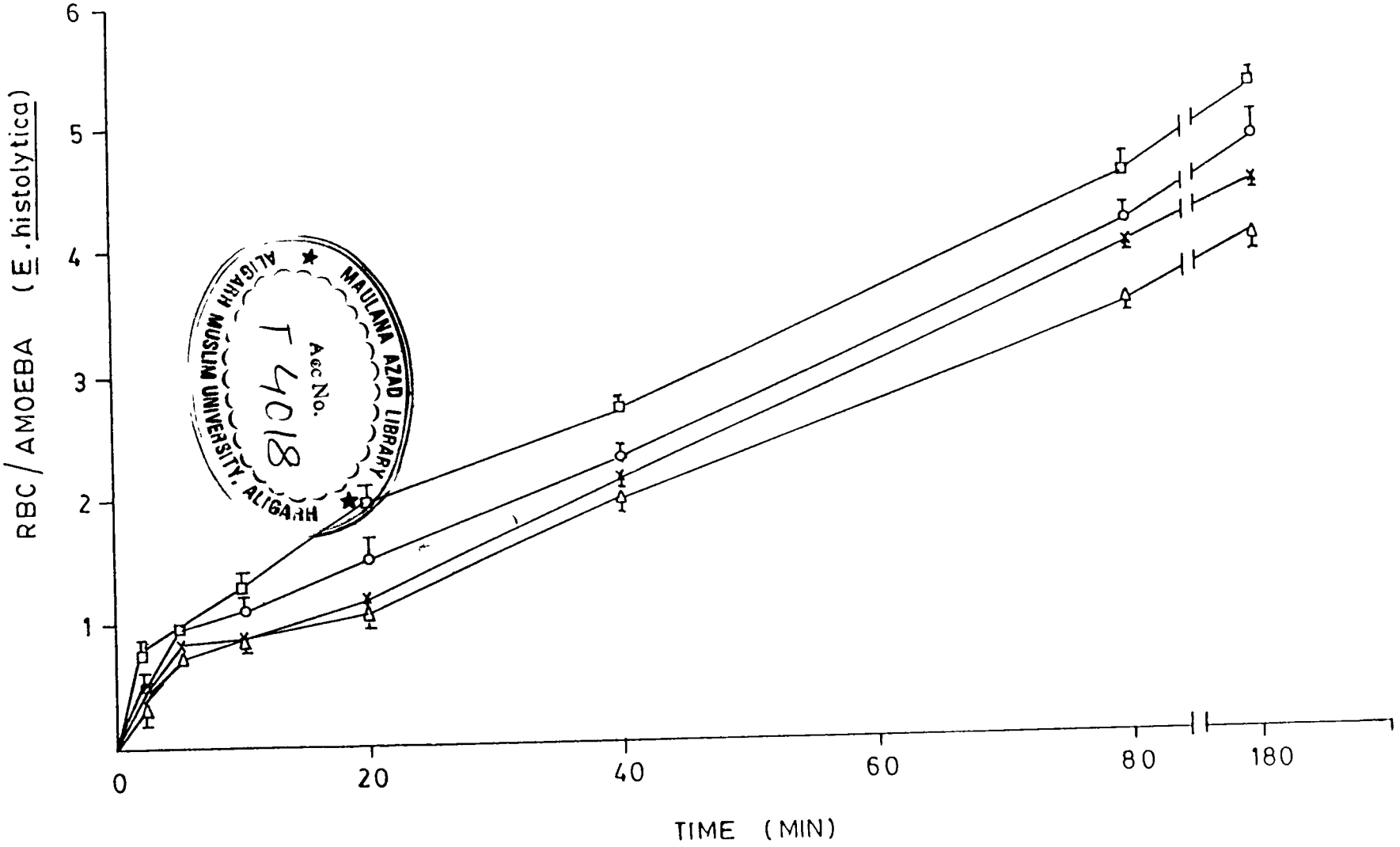


Fig.6. Comparison of RBC phagocytosis in the parent and the clonal cultures derived from DKB strain. Parent (o-o), DKB-1 (Δ-Δ), DKB-2 (x-x) and DKB-3 (□-□). Bars represent SD values obtained from three independent experiments.

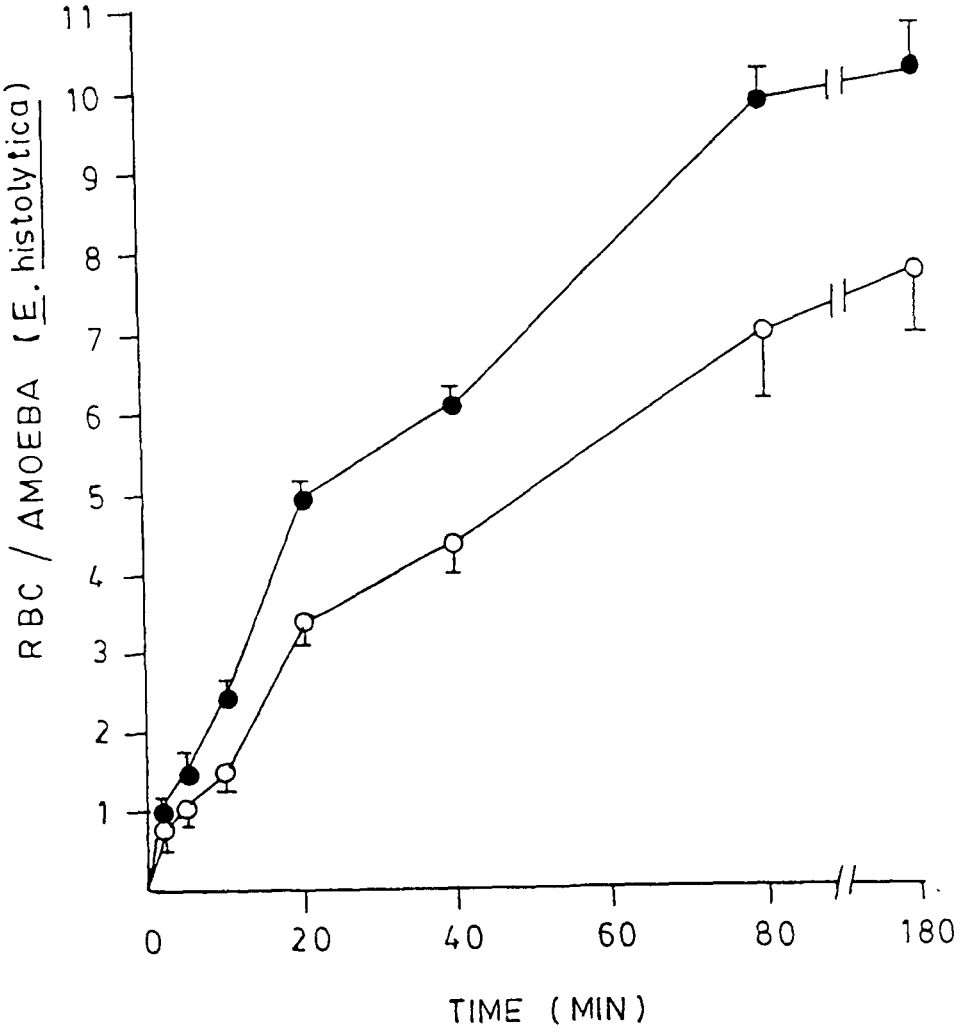


Fig.7. Influence of serial passage of **E.histolytica** (NIH:200) through cholesterol enriched medium on its erythrophagocytotic ability. Kinetics of erythrophagocytosis before (o-o) and after (●-●) cholesterol passage. Bars represent SD values obtained from three independent experiments.

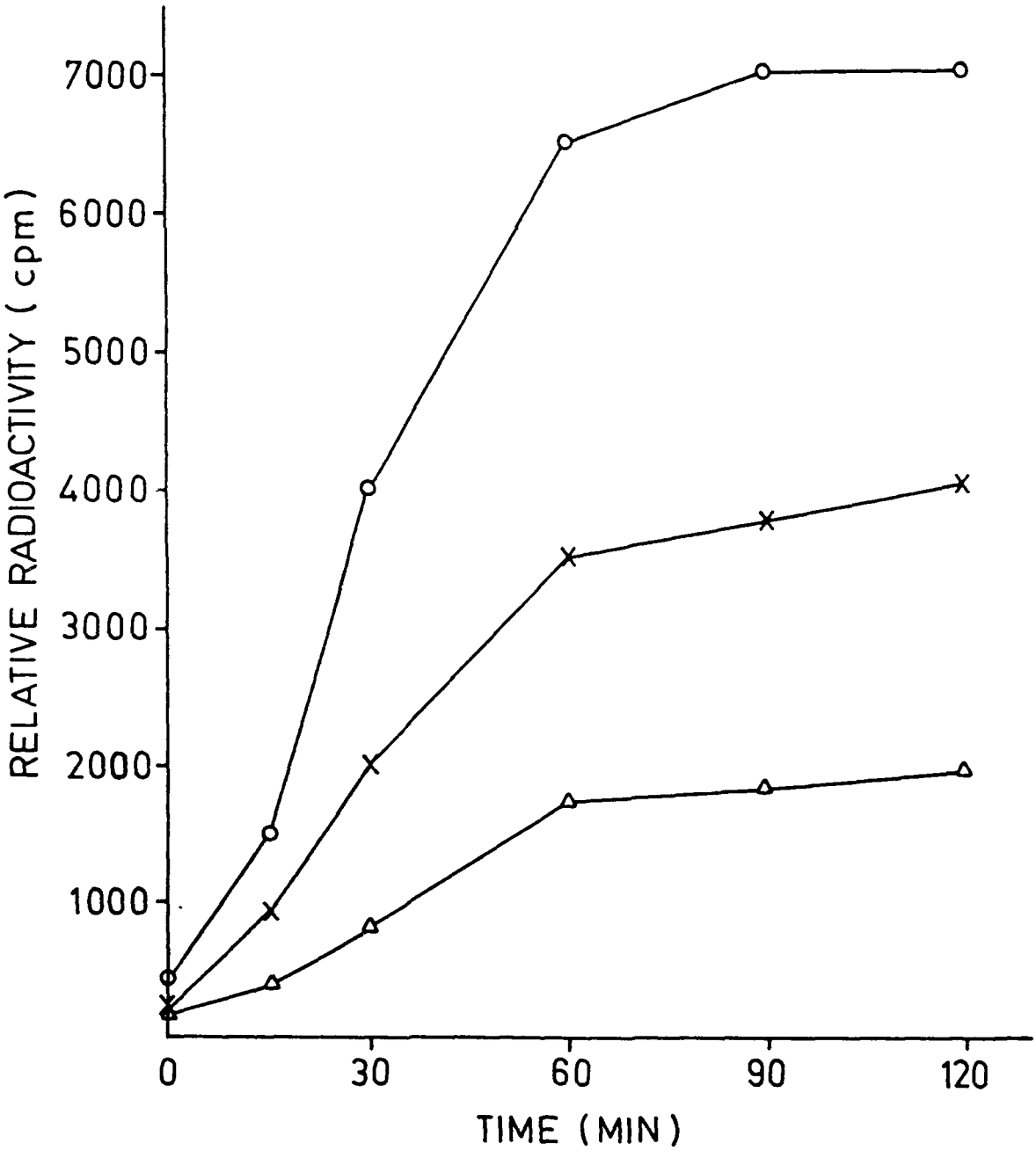


Fig.8. Comparison of ^{14}C-labelled RBC ghost phagocytosis (in terms of relative radioactivity) with time in IP:106 (o-o) NIH:200 (x-x) and DKB (Δ-Δ) strains of **E.histolytica**. Values represent average of two independent experiments.

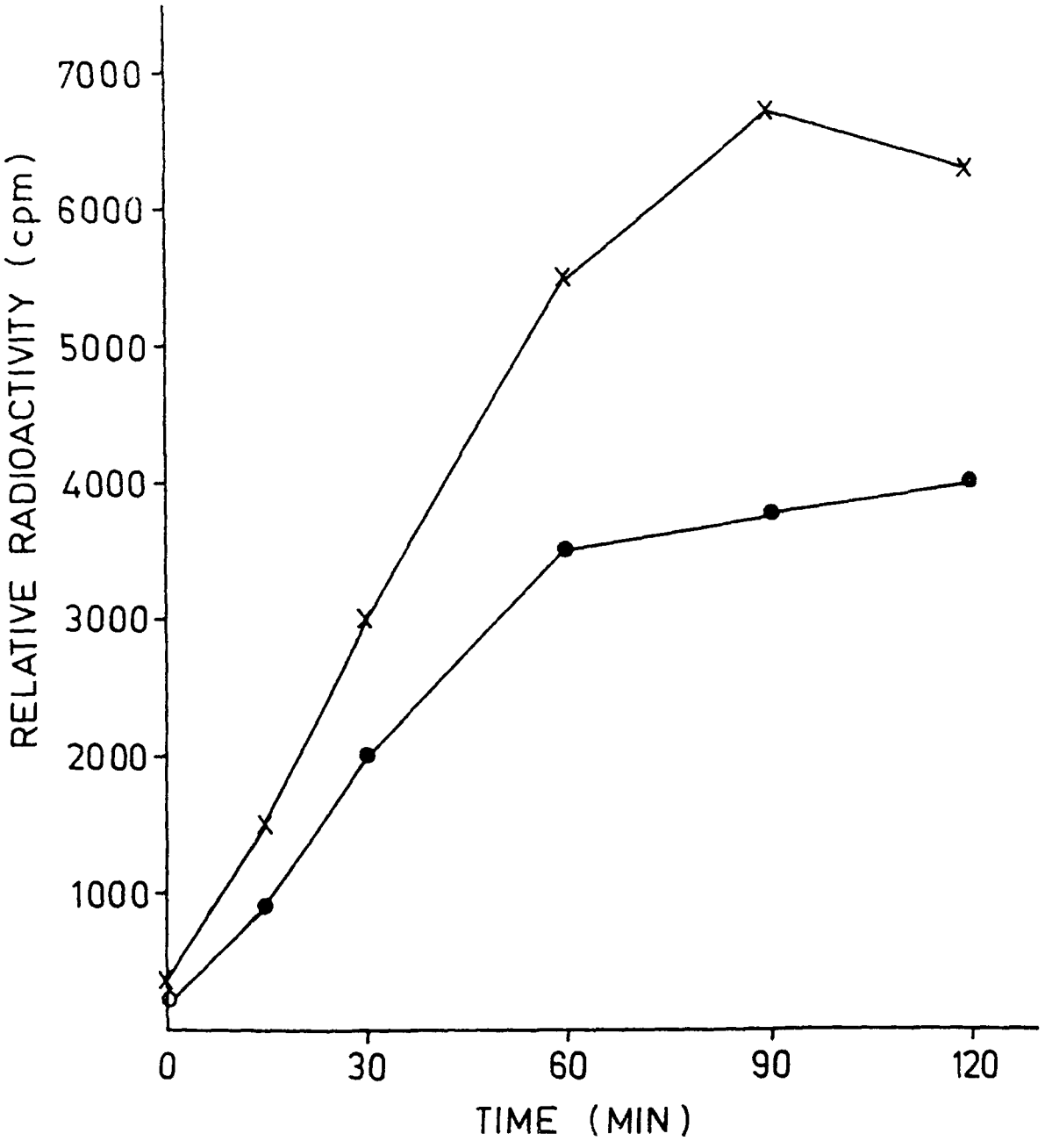


Fig.9. Influence of serial passage of NIH:200 strain of **E.histolytica** through cholesterol enriched medium on RBC ghost phagocytosis. NIH:200 (●-●), cholesterol passaged NIH:200 (x-x). Values represent average of two experiments.

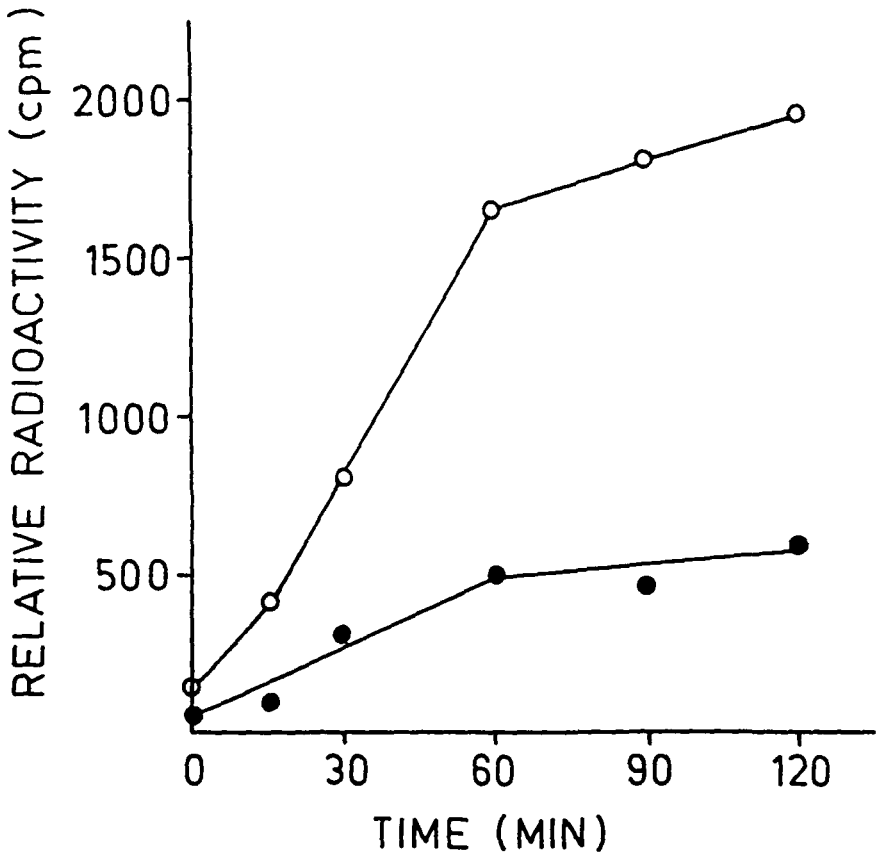


Fig.10. Effect of N-acetyl galactosamine treatment of **E.histolytica** (DKB) on RBC ghost phagocytosis. Without treatment (o-o), with treatment (●-●). Values represent average of two independent experiment.

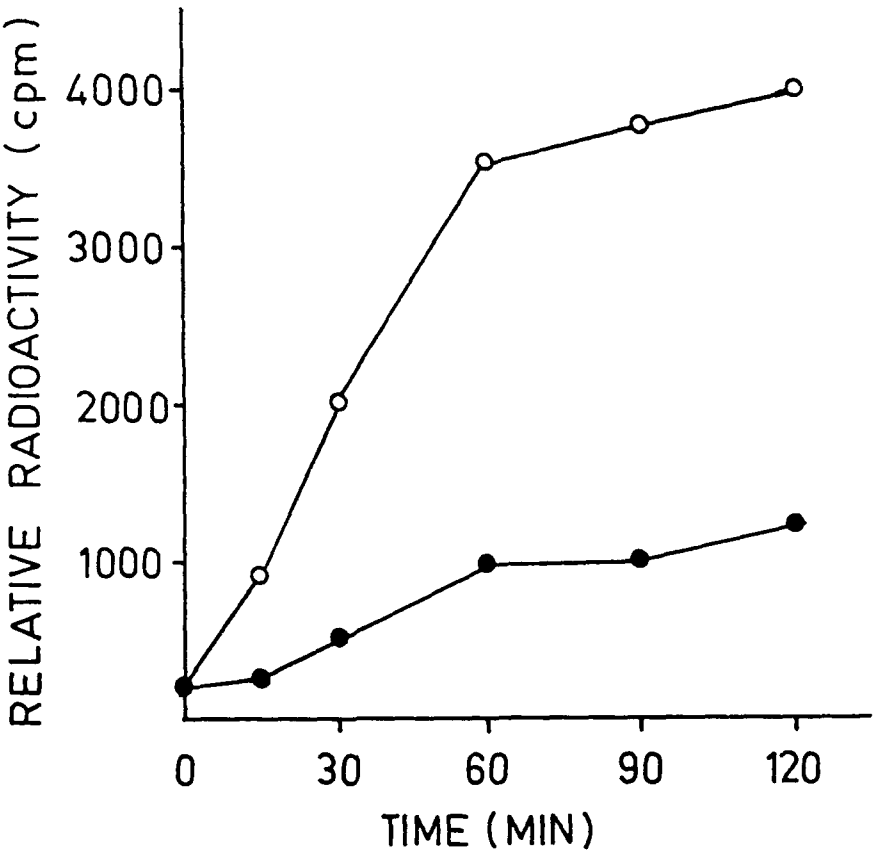


Fig.11. Effect of N-acetylgalactosamine treatment of **E.histolytica** (NIH:200) on RBC ghost phagocytosis. Without treatment (o-o), with treatment (•-•). Values represent average of two independent experiments.

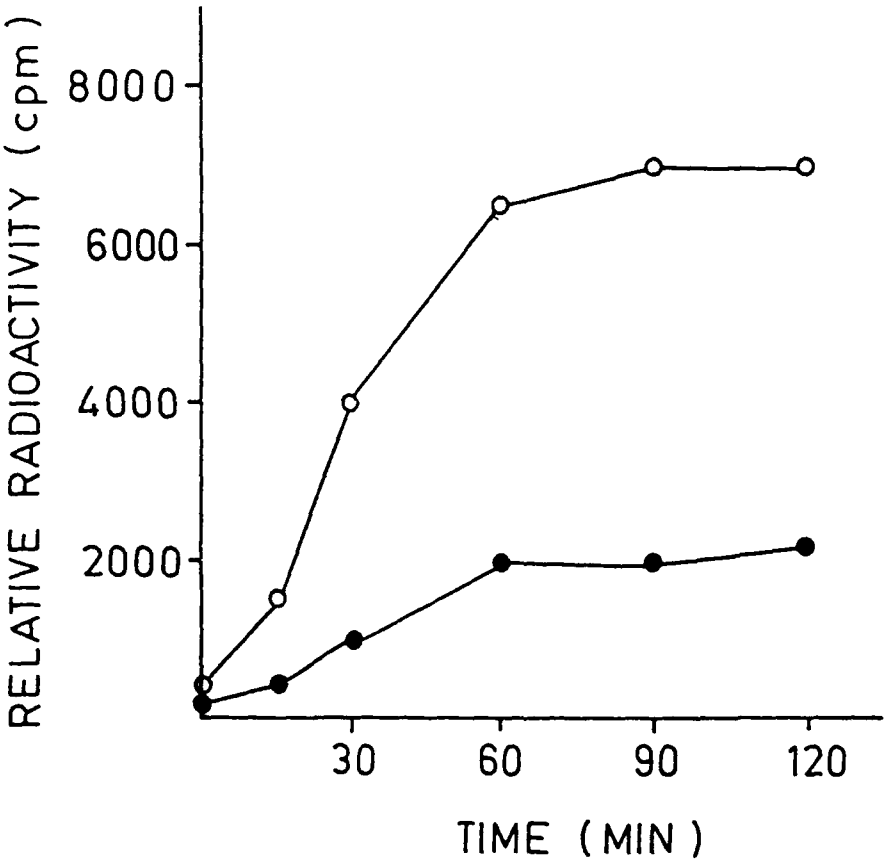


Fig.12. Effect of N-acetylgalactosamine treatment of **E.histolytica** (IP:106) on RBC ghost phagocytosis. Without treatment (o-o), with treatment (●-●). Values represent average of two independent experiments.

in both Hank's and Eagle's media. However, the substrates DL-serine, glucose and pyruvate did augment NBT reduction to different extents. Further, a much more pronounced increase in NBT reduction was induced both by NADH and NADPH (0.2 mM), the enhancement being more than two fold higher with the latter (Table 9).

Relative reduction of NBT by E.histolytica cells, homogenates and fractions

The results presented in Table 10 show a yeiled of about 57% formazan in the reaction mixture pellets when the activity was followed in the amoebic homogenate, relative to its total recovery from intact cells (100%). Further, bulk of this activity (more than 60%) was apparently localized in the fraction sedimentable at a very low speed of 600xg (0.6 p 15), whose microscopic examination showed large aggregates of membrane fragments/vesicles. The same low speed sedimentable fraction shared about two third ^{125}I radioactivity, when the cells were similarly homogenized and fractionated after surface labelling with Na ^{125}I. Significant amount of formazan was also produced from NBT by the supernatant fraction (24 S 30) although the conditions of its assay in this case were not strictly comparable (Table 10).

Effect of inhibitors/activators on NBT reduction

To understand the mechanism of NBT reduction by **E.histolytica**, the action of certain metabolic inhibitors/activators on the generation of formazan from this dye was examined. The results (Table 11)

showed that the reaction is highly sensitive to all the -SH blocking agents viz., iodoacetate, N - ethyl maleimide (NEM) and p-chloromercuric benzoate (pCMB). The EDTA and EGTA, on the other hand, were found to stimulate this activity under our experimental conditions, although o-phenanthroline (another metal chelator) produced inhibition. Fluoride, rotenone and antimycin A were almost ineffective, the latter being insensitive also towards the aerobic respiration of **E.histolytica**, as reported by Weinbach and Diamond (1974).

Effect of antiamoebic compounds on NBT reduction

Out of several tested antiamoebic compounds (emetine, metronidazole, quinacrine, iodochlorohydroxyquinoline, diloxanide furoate, diodoquin and phanquinone, quinacrine, an inhibitor of election transfer from NADH/NADPH to flavin moieties (Weinbach et al., 1980) and phanquinone (which was found by us to spontaneously and progressively oxidise both NADH and NADPH in complete absence of any biological entity) inhibited NBT reduction. Diodoquin, metronidazole and emetine were also inhibitory to NBT reduction (Table 12).

Effect of superoxide dismutase (SOD) and anaerobic environment on NBT reduction

To assess the possible role of superoxide radicals in the mechanism of electron transfer, the effect of SOD on the reduction of NBT was examined both in cell suspensions and homogenates of **E.histolytica**. Only about 8% curtailment in reduction of NBT to formazan was observed in intact cells at SOD concentration of 100 units/ml (possibly because

of low permeability of the enzyme or its competition with endogenous SOD of the amoeba). However, a much more pronounced inhibition of NBT reduction was noted with the homogenates; which increased progressively with SOD concentration, attaining a plateau at about 35% inhibition (Fig. 13). Nearly same degree of reduction (30%) was noticed in formazan production from NBT, when this was carried out under anaerobic conditions in tubes through which a stream of nitrogen was passed before initiating the reaction (Table 13).

Sensitivity to phorbol myristate acetate

Phorbol myristate acetate (PMA) which activate superoxide anion generation in polymorphoncuelar leukocytes (DeChatelet et al., 1976), also influences the NBT reduction in **E.histolytica** cells (Fig.14). Further, this increase in NBT reduction in intact trophozoites by PMA is almost insensitive to 100 units of SOD, when the two were supplied together (Fig. 14).

Sensitivity to H_2O_2 and catalase

Reduction of NBT by **E.histolytica** (as reported above) is sensitive to -SH blocking agents. Oxidative ability of H_2O_2 a product of **E.histolytica** respiratory activity, could thus, influence its own -SH functions and action of catalase and H_2O_2 on reduction of the dye was, therefore, investigated. The results showed significant reduction of formazan production from this dye by H_2O_2 while catalase induced an opposite response (Fig. 15). Further, catalase reverse the effect of H_2O_2 when two were supplied together (Fig. 15).

Effect of Concanavalin A (Con A)

Data showing action of Con A and its hapten ∝-methyl mannoside, on NBT reduction by the amoebic cells is shown in Fig. 16. These results show a concentration dependent stimulation of dye reduction by the lectin. Further, although ∝-methyl mannoside itself was found to induce a significant increase in NBT reduction, it curtailed the stimulatory action of the lectin when the two were supplied together. Superoxide dismutase in intact cells of **E.histolytica** also slightly curtailed the stimulatory effect of Con A (Fig. 17).

NBT reduction activity in different E.histolytica cultures

The comparative data on NBT reducing capacity of normal and cholesterol passaged NIH:200 and IP:106 are presented in Fig.18. These results show markedly higher specific activities of NBT reduction in IP:106 and significant stimulation of these activities in NIH:200 on its cultivation in cholesterol enriched environment. Such cholesterol treatment has been shown to augment also the protein content of NIH:200 cells (Katiyar et al., 1987), thus actual increase in the above activities per cell may be even greater than what is reflect in respective specific activity values.

Effect of erythrophagocytosis on NBT reduction

Phagocytosis of bacteria has been reported to enhance NBT reduction in **E.histolytica** (Aust-Kettis et al., 1982) as occurs also in erythrophagocytosis on the nitroblue tetrazolium reducing ability which was studied by comparing formazan production from this dye

by amoebae alone and amoebae-erythrocyte mixtures. The dimethyl sulphoxide extracts of equivalent quantity of erythrocytes alone did not show any significant change in their absorbance with time and gave an O.D. value of 0.117±0.008. The results after substracting this from the values obtained in case of amoebae-erythrocyte mixtures are presented in Fig. 19, alongwith similar data for equivalent quantities of amoebae alone. The results suggest a significant increase in the dye reductive ability of both IP:106 and NIH:200 cultures of **E.histolytica** in presence of erythrocytes, suggesting that erythrophagocytosis increases their capacity to reduce the dye.

OXYGEN UPTAKE STUDIES

Endogenous oxygen uptake ($E\text{-}QO_2$) in **E.histolytica** in presence of RBC and Escherichia coli (native and heat killed) are presented in Table 14. The data shows that there is no stimulation of oxygen uptake by RBC, although it significantly increases the NBT reduction in **E.histolytica**. Further, heat killed E.coli shows very little effect on this property of **E.histolytica** while native E.coli increases it upto 150.0%.

STUDIES ON ALCOHOL DEHYDROGENASE

Alcohol is one of the major end products of carbohydrate metabolism in **E.histolytica** and is generated through the mediation of alcohol dehydrogenase. This enzyme has been considered to play an important role in **E.histolytica** in the regeneration of NAD (from glycolytically reduced coenzyme) and thus maintains continued operation

of the glycolytic cycle. Our experiments showed that the optimum pH of this enzyme in backward direction is 6.5 (Fig. 20). The equilibrium of this enzyme reaction in **E.histolytica** highly favoured production of alcohol from acetaldehyde and the backward reaction had a very poor rate. Further, the enzyme showed a much higher activity in presence of NADH as compared to NADPH (Table 15).

A comparison of the specific activity of this enzyme in NIH:200 and IP:106 cultures gave more than two fold higher activity in the latter (Table 15). Further, this activity in NIH:200 culture showed marked increase when the amoebae were cultivated in cholesterol enriched medium.

Effect of inhibitors

Results showing the sensitivity of alcohol dehydrogenase to various inhibitors in IP:106 and normal and cholesterol passaged NIH:200 strains of **E.histolytica** are presented in Table 16. The data show that this enzyme activity was also sensitive to -SH group blocking agents, pCMB in this case being more potent. A very strong inhibition of this enzyme activity was observed with Zn^{2+}, borate and Mg^{2+} ions. EGTA, EDTA, O-phenanthroline, imidazole and β-mercaptoethanol were also inhibitory to this activity. However, the pattern of effect of the compounds tested was almost similar in different cultures.

Effect of antiamoebic compounds

Amongst the several tested antiamoebic compounds listed in Table 17, only diloxanide furoate was slightly inhibitory to this enzyme.

Table 8: Formazan production by **E.histolytica** (NIH:200) in different medium/buffer.

Medium/Buffer	Formazan production (O.D. at 572 nm,(mg protein)$^{-1}$)			
	30 min	60 min	90 min	120 min
Hank's buffer	0.202	0.306	0.381	0.452
Phosphate buffer	0.196	0.296	0.372	0.438
Normal saline	0.171	0.268	0.349	0.402
Eagle's medium	0.208	0.312	0.396	0.471

All values are mean of two independent experiments.

Table 9: Effect of exogeneous substrates/coenzymes on NBT reduction by **E.histolytica** (NIH:200).

Substrate/coenzyme	Concentration (mM)	Relative activity
Control (Hank's balanced salt solution)	-	100.0
Glucose	0.5	117.7
Pyruvate	0.1	138.2
DL-Serine	0.5	164.2
NADH	0.2	810.0
NADPH	0.2	1991.0
NAD	0.2	101.9
NADP	0.2	98.4

All data are average of the values obtained in two independent experiments.

Table 10: NBT reducing activity of **E.histolytica** (NIH:200) cells, homogenates and cellular fractions.

Activity source	Relative NBT→Formazan conversion (OD change/ 3.3×10^6 cells at 572 nm)	Relative ^{125}I label (%)
Cell suspension	1.079	100.0
Homogenate	0.612	100.0
0.6 p 15	0.383	66.7
24 p 30	0.094	16.0
24 S 30	0.254[a]	10.4

All data are average of the values obtained in two independent experiments.

[a]Formazan produced by (24 S 30) was released in the aqueous medium and was thus not extractable with DMSO. This was assessed against appropriate zero time aqueous control, without extraction.

Table 11: Effect of inhibitors/activators on reduction of NBT by *E.histolytica* (NIH:200) cells.

Inhibitors/Activators	Concentration (mM)	Relative activity
Control	-	100.0
Iodoacetate	1.0	35.5
	2.0	10.6
pCMB	0.5	22.6
	1.0	7.6
NEM	1.0	43.5
	3.0	12.5
Sodium fluoride	1.0	92.4
	2.0	88.5
O-Phenanthroline	3.0	22.6
	6.0	16.9
EGTA	1.0	213.2
	2.0	258.2
EDTA	1.0	232.0
	2.0	296.4
Antimycin A	1.0	100.0
	2.0	100.0
Rotenone	0.1	99.0
	0.2	98.6
Deoxycholate	0.06%	64.7
	0.12%	32.6
Boiled	-	0.0

All data are average of two independent experments.

Table 12: Effect of antiamoebic compounds on NBT reduction in NIH:200 strain of **E.histolytica**.

Compounds	Concentration (mM)	Relative activity
Control	-	100.0
Emetine HCl	0.1	64.7
	0.2	32.6
Metronidazole	0.1	81.6
	0.2	78.9
Quinacrine	0.1	87.5
	0.2	35.9
Iodochlorohydroxyquinoline	0.1	98.8
	0.2	89.9
Diloxanide furoate	0.1	96.7
	0.2	90.3
Diodoquin	0.1	87.5
	0.2	78.3
Phanquinone	0.02	57.0
	0.04	52.9
	0.1	49.5

All data are average of two independent experiments.

Table 13: Effect of anaerobic condition on NBT reduction by NIH:200 strain of **E.histolytica**.

Conditions	NBT→ Formazan conversion (OD change/3.3×10^6 cells at 572 nm)	Relative activity
Aerobic	1.145	100.0
Anaerobic*	0.802	70.0

*To create anaerobic conditions in samples, stream of nitrogen was passed before initiating the reaction.

Data are average values of two independent experiments.

Table 14: Oxygen uptake by **E.histolytica** (NIH:200) and its stimulation by Escherichia coli/RBC.

Source	O_2 uptake n atom/min	% Increase
E.histolytica (1.0×10^6 cells/ml)[a]	144.00	(100.0)
E.coli (1.0×10^8 cells/ml, heat killed)[b]	0.00	-
E.coli (1.0×10^8 cells/ml, native)[c]	79.24	-
E.coli (2.0×10^8 cells/ml, native)[d]	140.00	-
RBC (1.0×10^8/ml)[e]	17.50	-
E.histolytica[a] + E.coli[b]	154.40	(106.9)
E.histolytica[a] + E.coli[c]	400.00	(222.7)
E.histolytica[a] + E.coli[d]	500.00	(250.0)
E.histolytica[a] + RBC[e]	168.20	(104.6)

Data in parenthesis shows the relative values of oxygen uptake by **E.histolytica** when bacteria/RBC added (after substracting the corresponding oxygen uptake values of E. coli/ RBC).

All data are average of two independent experiments.

Table 15: Activities of alcohol dehydrogenase in the cell extracts of axenic NIH:200 (normal and cholesterol passaged) and IP:106 strains of **E.histolytica**.

Reaction direction	Coenzyme supplied	Specific activity (nmoles product formed min^{-1} $mg\ protein^{-1}$)		
		NIH:200	NIH:200 (Cholesterol passaged)	IP:106
Backward direction (acetaldehyde → alcohol)	NADH	327.0±14.0	587.0±2.0	693.0±88.0
	NADPH	140.0±6.0	267.0±12.0	330.0±44.0
Forward direction (alcohol→ acetaldehyde)	NAD	18.0±3.0	34.0±4.0	38.0±7.0
	NADP	50.0±3.0	76.0±11.0	74.0±7.0

Values are mean ± S.D. based on data obtained in 3 independent experiments.

Table 16: Effect of inhibitors on NADH dependent alcohol dehydrogenase activity (backward direction) in different strains of **E.histolytica**.

Inhibitors	Concentration (mM)	Relative activity		
		NIH:200	NIH:200 (Cholesterol passaged)	IP:106
Control	-	100.0	100.0	100.0
EDTA	1.0 , 2.0	55.7, 51.1	53.6, 49.9	64.1, 49.5
pCMB	0.01, 0.04	4.6, 0.0	0.0, 0.0	0.0, 0.0
Imidazole	1.0, 2.0	46.6, 9.2	49.9, 20.7	64.9, 33.6
Deoxycholate	1.0, 2.0	31.2, 30.5	54.2, 45.6	39.9, 30.6
EGTA	0.4, 2.0	66.5, 55.5	60.7, 35.7	64.1, 58.3
O-Phenanthrolene	0.4, 2.0	55.5, 38.7	62.4, 33.3	75.0, 39.0
β-Mercaptoethanol	1.0, 4.0	72.1, 55.5	45.8, 45.8	89.9, 69.8
Iodoacetate	1.0, 2.0	41.7, 9.2	33.3, 16.6	54.5, 14.6
NEM	1.0, 2.0	44.4, 40.0	41.5, 36.5	39.9, 35.4
Sodium borate	0.4, 0.8	37.4, 21.1	39.7, 16.4	41.6, 17.0
DTT	0.2, 2.0	60.9, 95.5	66.6,104.0	79.9, 94.8
$ZnSO_4$ (Zn^{2+})	0.4, 1.0	22.2, 16.6	20.7, 0.0	0.9, 0.0
$MgCl_2$ (Mg^{2+})	1.0, 2.0	72.3, 65.6	75.0, 63.0	69.8, 60.5

All data are average of two independent experiments.

Table 17: Effect of antiamoebic compounds on NADH-dependent alcohol dehydrogenase activity (backward direction) of **E.histolytica** (NIH:200).

Antiamoebic compounds	Concentration (μM)	Relative activity
Control	-	100.0
Diloxanide furoate	10	70.0
	20	66.6
Quinacrine	10	90.0
	20	86.6
Iodochlorohydroxyquinoline	10	96.0
	20	92.5
Diodoquin	10	91.5
	20	82.5
Phanquinone*	-	-
Metronidazole*	-	-

*Spontaneously and progressively oxidise both NADH/NADPH in complete absence of any biological entity.

Data are average of two independent experiments.

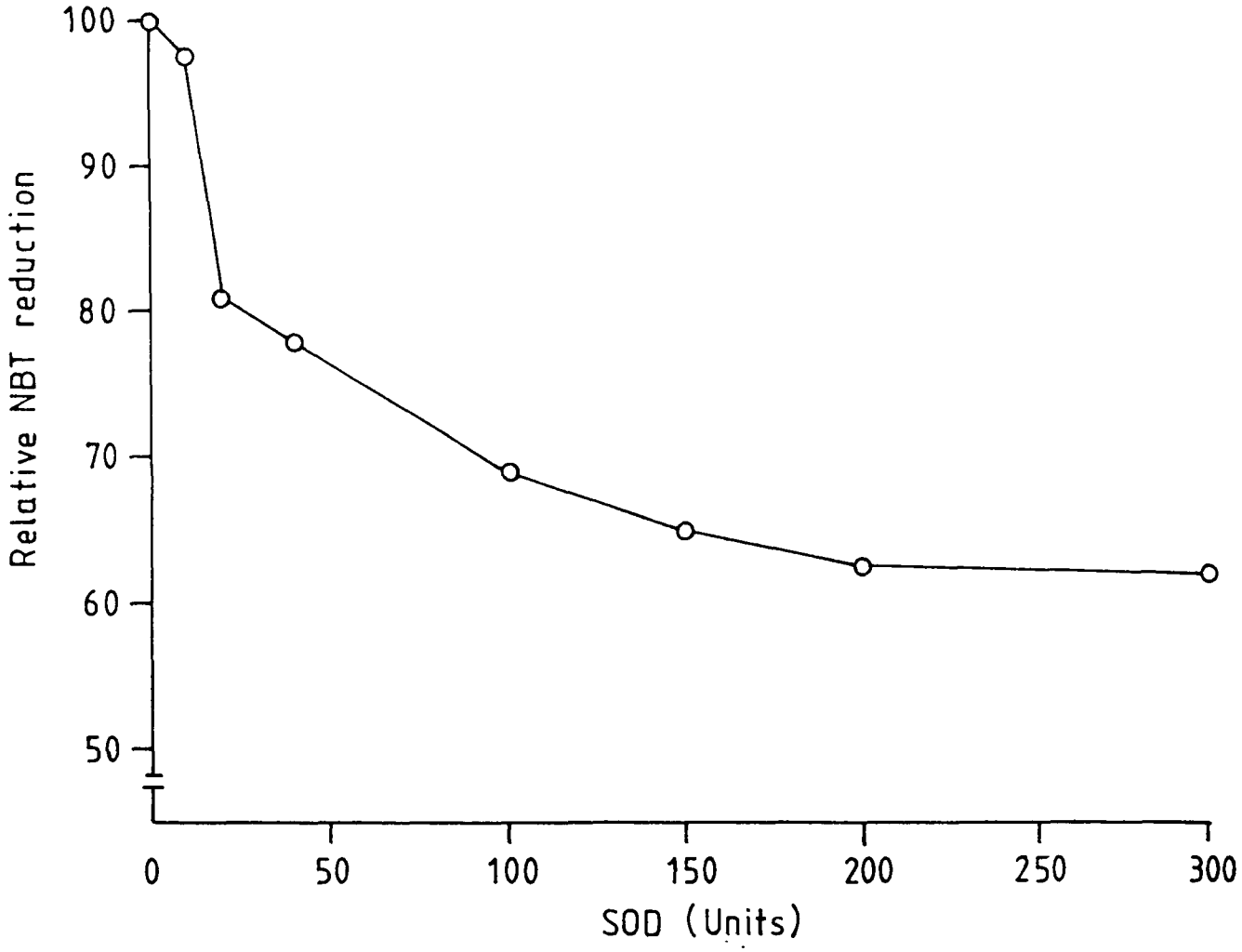


Fig.13. Effect of superoxide dismutase (SOD) on NBT reduction of **E.histolytica** (NIH:200). Values represent average of two independent experiments.

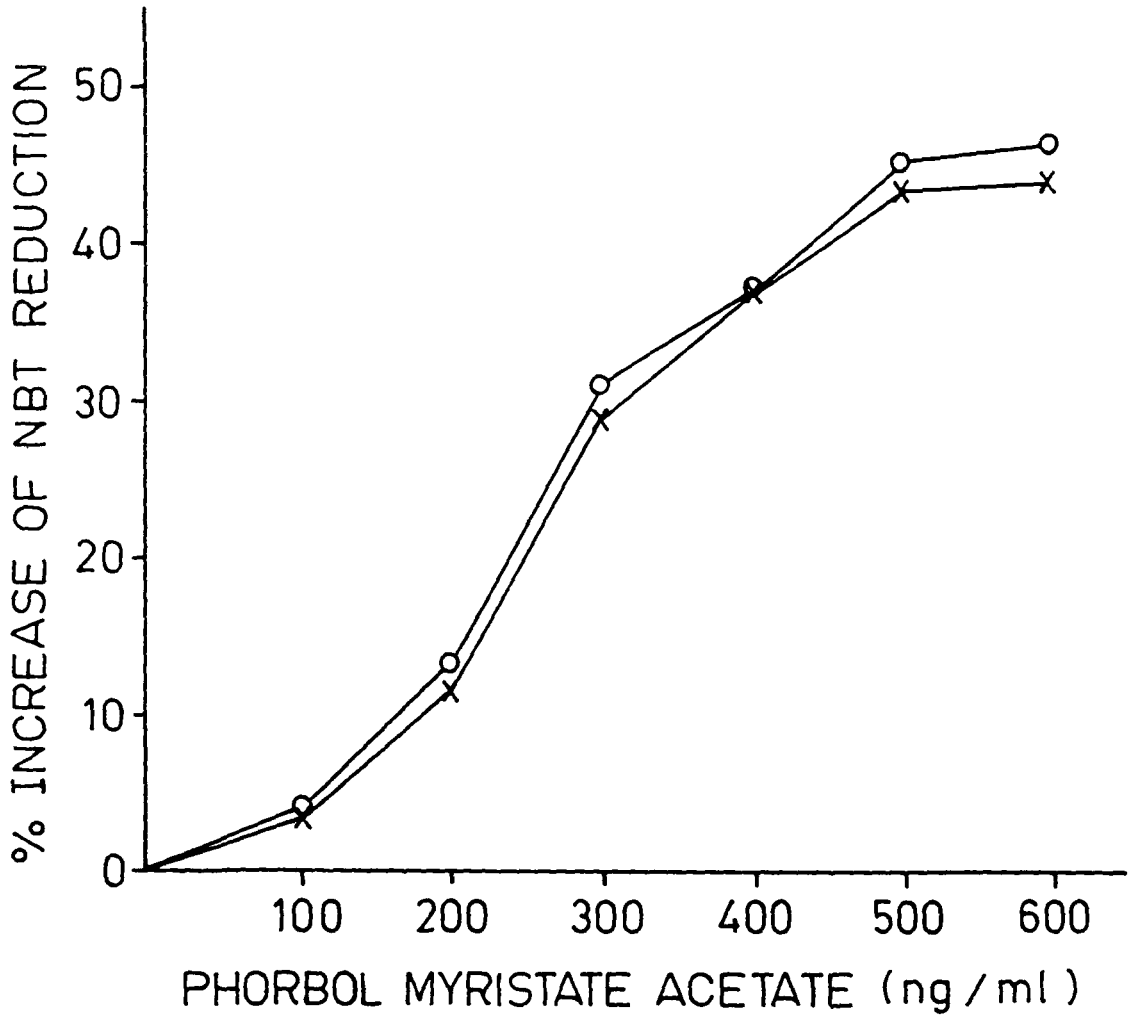


Fig.14. Stimulation of NBT reduction of NIH:200 strain of **E.histolytica** (in terms of % increase) by phorbol myristate acetate (o-o) and effect of 100 units of superoxide dismutase (x-x) on this stimulation. Data represent average of two independent experiments.

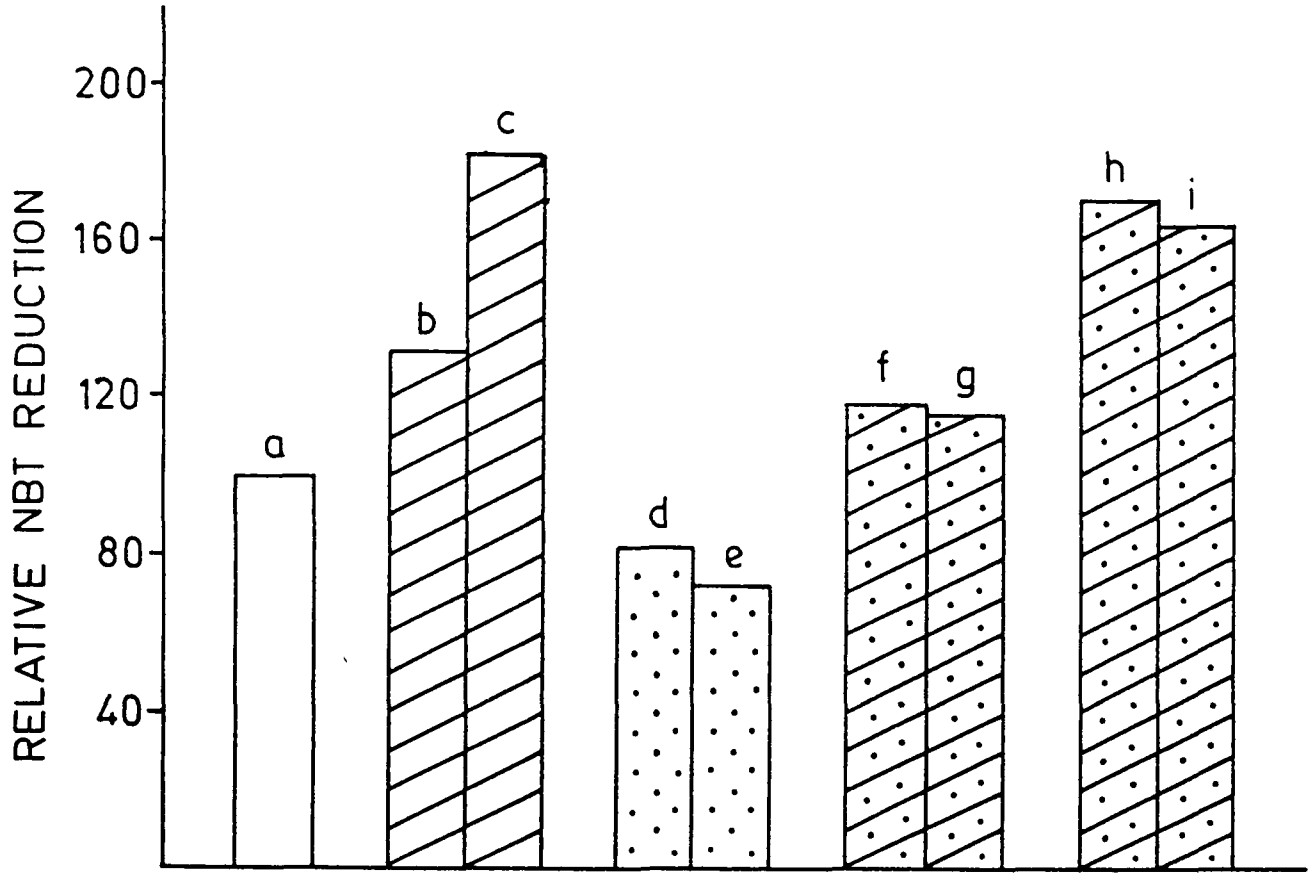


Fig.15. Influence of catalase and Hydrogenperoxide on NBT reduction of **E.histolytica** (NIH:200). a : Control; b: +Catalase (2000 U); c: +Catalase (4000 U); d : + H_2O_2 (0.5 mM); e : + H_2O_2 (1.0 mM); f : + Catalase (2000 U) and H_2O_2 (0.5 mM); g : + Catalase (2000 U) and H_2O_2 (1.0 mM); h : + Catalase (4000 U) and H_2O_2 (0.5 mM); i : + Catalase (4000 U) and H_2O_2 (1.0 mM).

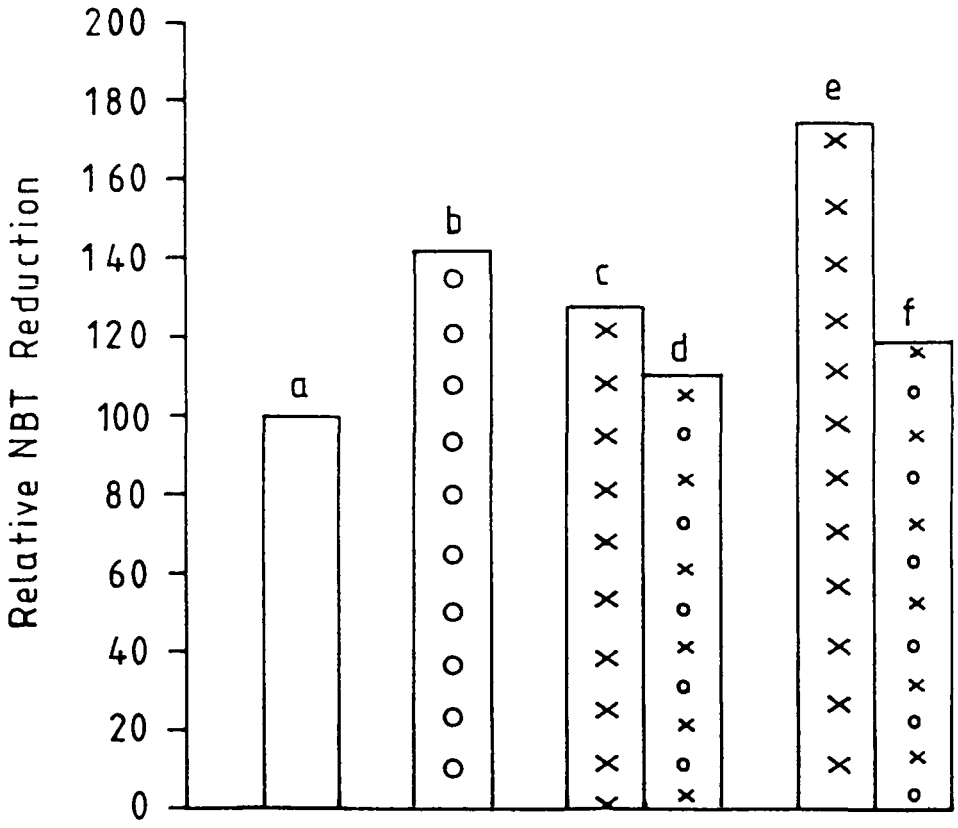


Fig.16. Influence of Con A on NBT reduction by **E.histolytica** (NIH:200) a : Control; b : + αC-methyl mannoside (100 mM); c : + Con A (50 µg/ml); d : + Con A (50 µg/ml) and αC-methyl mannoside (100 mM); e : + Con A (100 µg/ml); f : + Con A (100 µg/ml) and αC-methyl mannoside (100 mM).

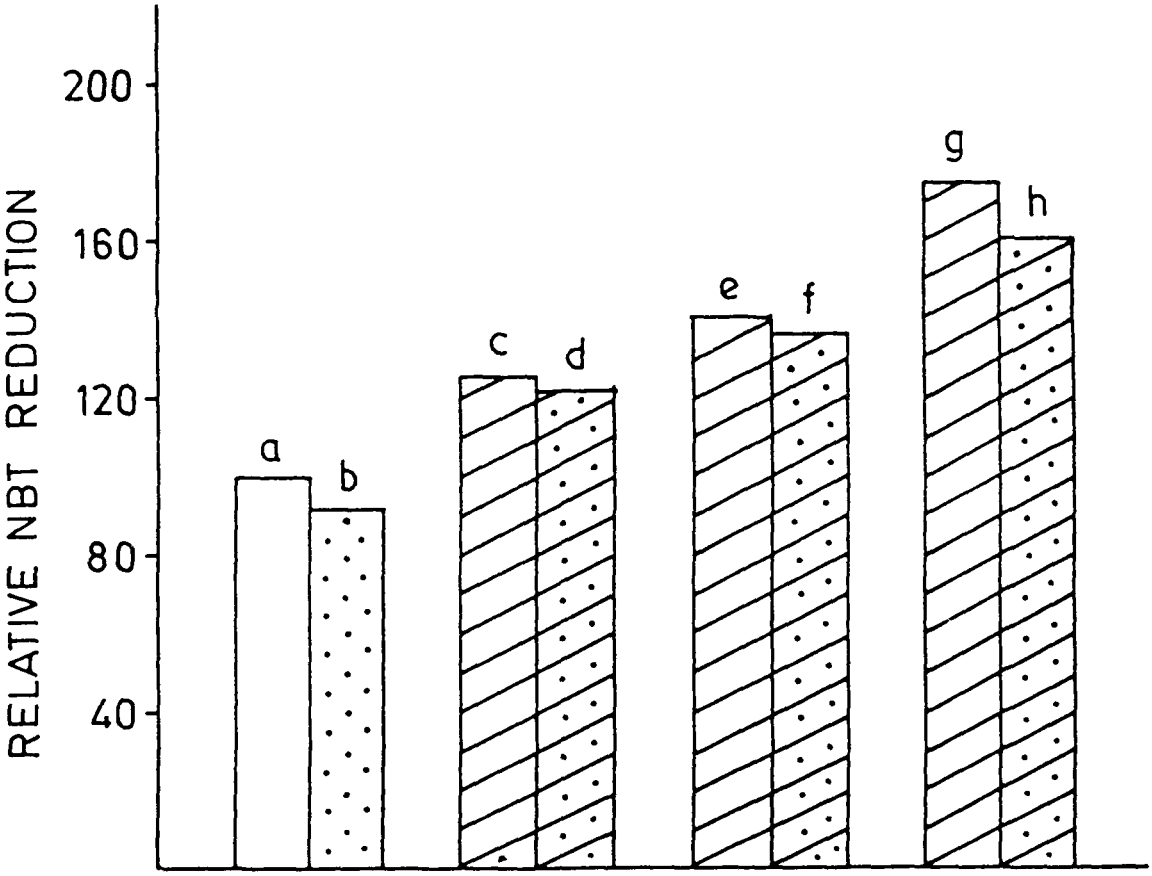


Fig.17. Influence of SOD on Con A stimulation of NBT reduction in **E.histolytica** (NIH:200). a : Control; b : + SOD (100 U); c : + Con A (50 µg/ml); d : + Con A (50 µg/ml) and SOD (100 U); e : + Con A (75 µg/ml); f : + Con A (75 µg/ml) and SOD (100 U); g : + Con A (100 µg/ml); h : + Con A (100 µg/ml) and SOD (100 U).

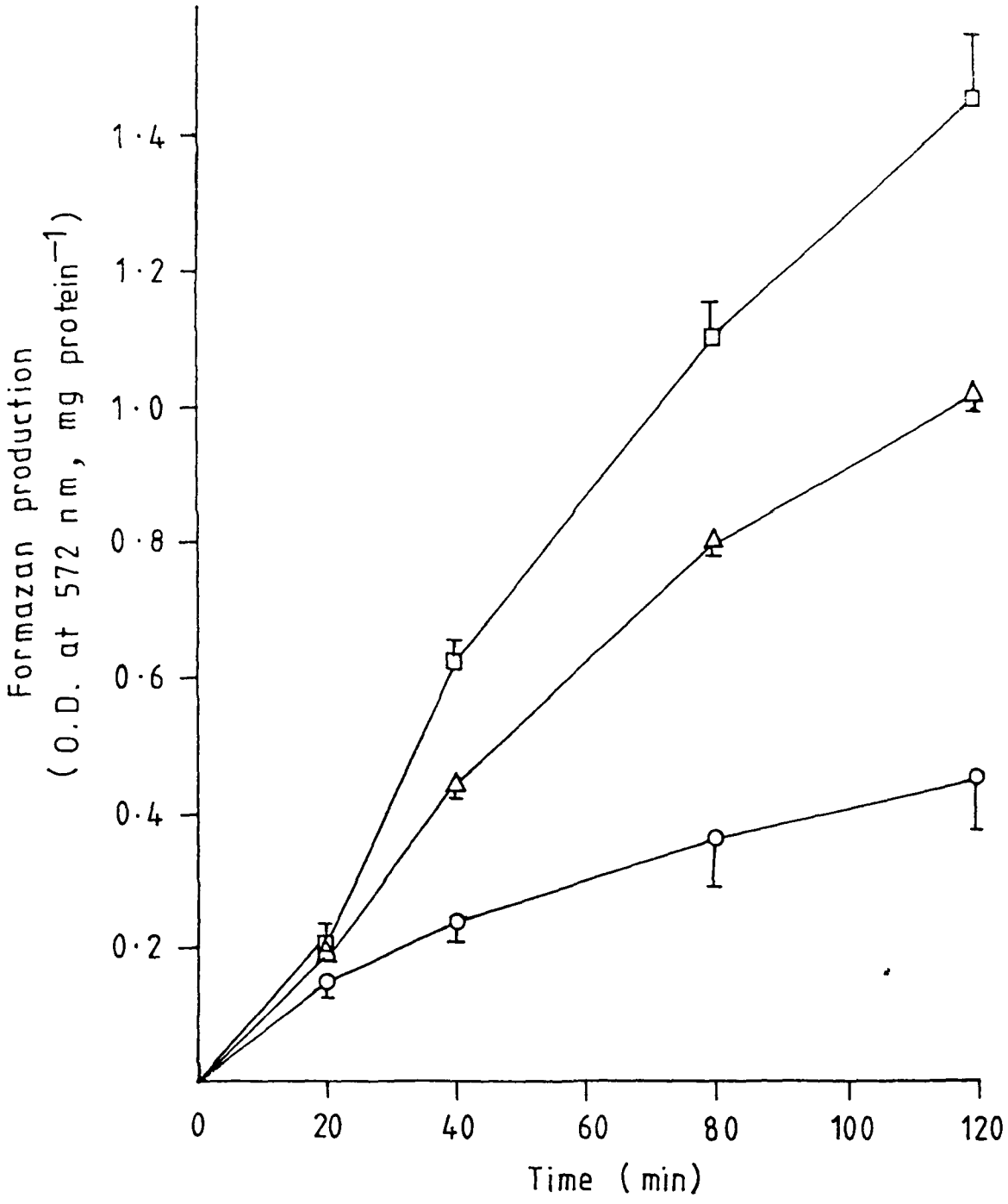


Fig.18. Specific NBT reducing activities (per mg protein) of different *E.histolytica* cultures. o-o, NIH:200; Δ —Δ , NIH:200 (Cholesterol passaged); □ -□ , IP:106; plotted values represents average of 3 experiments; bars represent SD values.

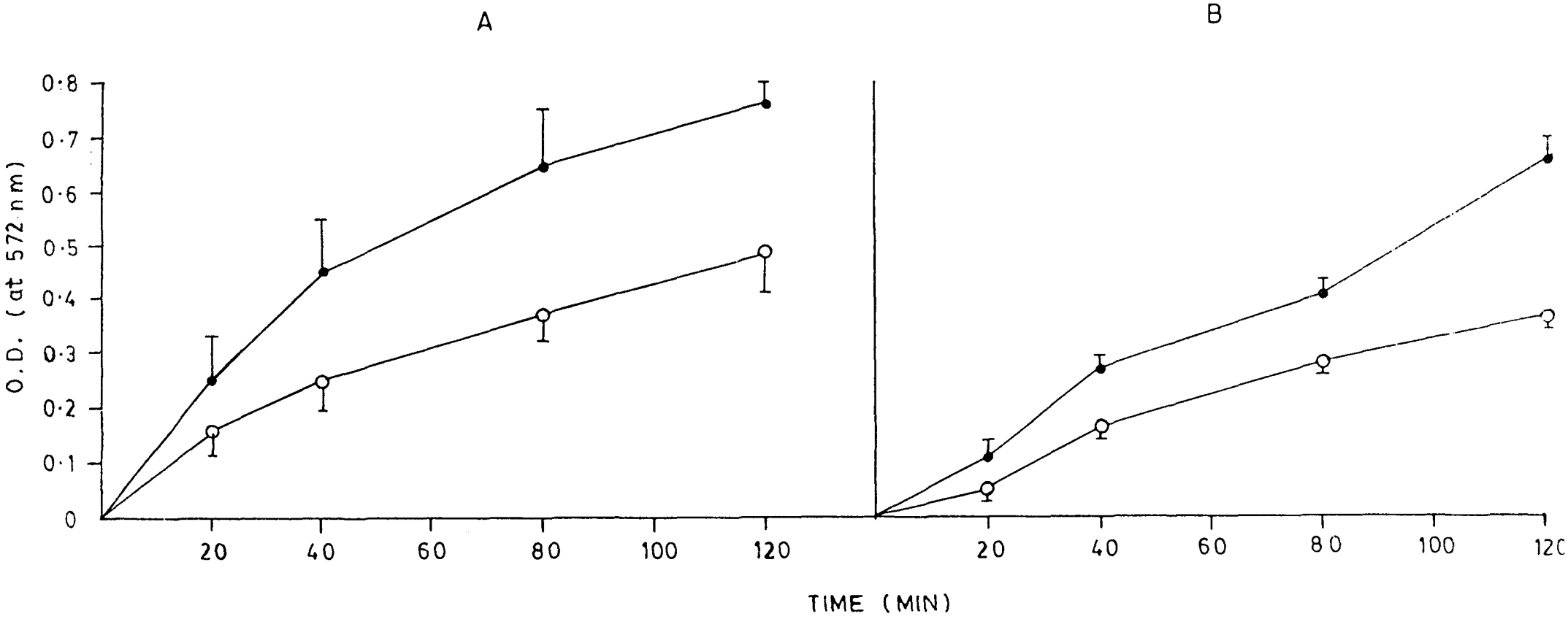


Fig.19. Enhancement in NBT reduction due to erythrophagocytosis by IP:106 (A) and NIH:200 (B) strains of **E.histolytica**. NBT reduction by **E.histolytica** cells alone (o-o) and in presence of erythrocytes (o-o).

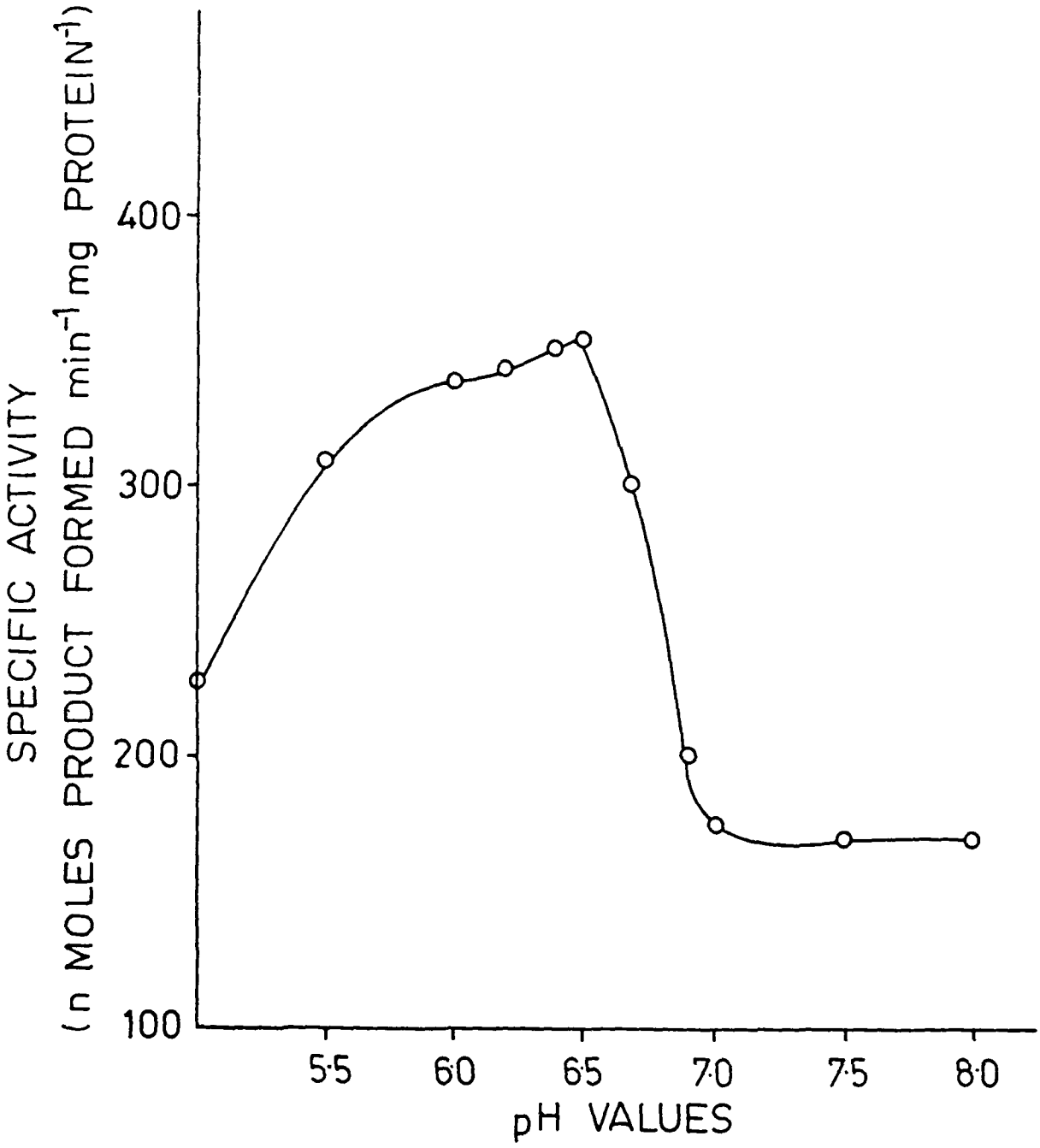


Fig.20. pH profile of alcohol dehydrogenase (backward reaction, acetaldehyde → ethanol) of **E.histolytica** (NIH:200).

Effect of phanquinone and metronidazole could not be confirmed because of spontaneous oxidation of both NADH/NADPH, by these drugs, in complete absence of biological entity.

C. ACID PHOSPHATASE (INTRACELLULAR AND EXTRACELLULAR) AND SULFHYDRYL PROTEINASE OF E.HISTOLYTICA

ACID PHOSPHATASE OF E.HISTOLYTICA

Acid phosphatase activity of **E.histolytica** has been claimed to show a correlation with its virulence (Eaton et al., 1969, 1970; Lal and Garg, 1979) and this enzyme has been used as a marker to identify surface active lysosomes in this amoeba. It was, therefore, considered of interest to investigate some properties of this enzyme.

Studies on acid phosphatase in Leishmania donovani have indicated that, as in **E.histolytica**, this enzyme is associated with both the plasma membrane and intracellular vacuoles (Gottlieb and Dwyer, 1981). In addition, the parasite has been found to secrete it in the cell free medium (Gottlieb and Dwyer, 1982). The present study reports several characteristics of intracellular and extracellularly secreted acid phosphatase of **E.histolytica**.

Comparative data on intracellular acid phosphatase activity in the homogenates of normal and cholesterol passaged NIH:200 & IP:106 strains of **E.histolytica** are presented in Table 18. These results show markedly higher specific activity of acid phosphatase in IP:106 and significant stimulation of this activity in NIH:200 on its cultivation in cholesterol ennriched environment.

Comparative data showing total acid phosphatase content of **E.histolytica** cell (as assayed in its homogenates) versus the enzyme activity released in the growth medium at different post inoculation intervals in the presence or absence of 0.1% triton X-100 are presented in Table 19. These results indicate progressive increase both in cell number and total enzyme content of harvested amoebic populations with time, as well as enzyme activity released in the medium. These enzymes content per cell did not show much variation with the age of the culture, however total extracellular levels of the enzyme were several fold higher as compared to the activity of the cellular populations at different post-inoculation intervals. Triton X-100 did not significantly influence the enzyme activity in either cellular homogenates or cell free medium.

Fractionation of the cell free medium obtained from 72 h old cultures by precipitation with $(NH_4)_2SO_4$ showed that the enzyme distributed itself in the fractions obtained at 0-20%, 20-40%, 40-60% salt saturation more or less in proportion to their protein contents. The specific activity values in these three fractions were 6.17, 6.53, 6.32, respectively, but declined to 3.19 in the fraction obtained at 60-80% $(NH_4)_2SO_4$ saturation.

DEAE-cellulose chromatography

The above three fractions were pooled together and loaded on a DEAE-cellulose column. Elution of this column with tris HCl buffer, pH 7.5 (0.1M) did not yield any enzyme activity although protein was eluted out (Fraction 1-17, Fig. 21). Further, stepwise

elution with 0.05,0.1,0.2M NaCl resulted in three sharp peaks of both the protein and enzyme as shown in the Fig. 21. The relative enzyme specific activities of the initial 0-60% saturation $(NH_4)_2SO_4$ precipitate and the pooled fraction of peaks AP_1, AP_2 and AP_3, alongwith the data showing extent of purification achieved in these peaks are presented in Table 20.

The PAGE of the enzyme (Table 20) in AP_1 gave 1 enzyme band (Rm: 0.46), 2 bands in AP_2 (Rm: 0.46 and 0.61) and 3 bands in AP_3 (Rm: 0.46, 0.64, 0.66).

Relative sensitivity of the acid phosphatase recovered in these peaks to some known inhibitors/activators of acid phosphatase or other biochemical functions and to some antiamoebic drugs are presented in Table 21. The enzyme in all these fractions showed insensitivity to tartarate but was inhibited by fluoride, Cu^{2+}, EDTA, ammonium molybdate and surprisingly also by cysteine The Concanavalin A, however, caused a concentration dependent inhibition of the enzyme in AP_1 but stimulated this activity in AP_2 and AP_3 at $0.4x10^{-6}$M and $0.4x10^{-5}$M lectin (MW of Con A: 25,000) concentration respectively. Both stimulatory and inhibitory responses showed reversal when glucose (2 mg/ml) was simultanenously added with the lectin. All these peaks showed presence of carbohydrate as tested by the phenol sulfuric acid method (Montgomary, 1957). Further, all the antiamoebic drugs tested were found to be inhibitory towards this enzyme activity of **E.histolytica**.

STUDIES ON SULFHYDRYL PROTEINASE (CYSTEINE PROTEINASE) OF E.HISTOLYTICA

Sulfhydryl proteinase activity of **E.histolytica** has been claimed to show a correlation with its virulence (Lushbaugh et al., 1985). It was, therefore, considered of interest to compare the activity and properties of this enzyme in different cultures of **E.histolytica**.

Effect of triton X-100

In earlier studies by McLaughlin and Faubert (1977), triton was used as a constituent of reaction mixture but no results were presented by them on the effect of the detergent on this activity of **E.histolytica**. Results showing relative proteinase activity in the absence and presence of 0.5% triton in homogenate and fractions of **E.histolytica** cultures are presented in Table 22. These results show that triton X-100 significantly increases this activity in Entamoeba homogenate and pellets while does not alter this in the 15,000xg supernatant fraction.

Effect of pH

The sharp pH optimum was obtained at pH 6.0 (Fig. 22) indicating this activity to be of neutral sulfhydryl proteinase (described by McLaughlin and Faubert, 1977).

Activity in homogenate and fractions in different cultures of E.histolytica

E.histolytica did show significant proteinase activity using azocasein as the substrate. The results in Table 22 show that the

major portion of this activity is localized in high speed sedimentable (10,000xg, 20 min) pellet, supernatant fraction contain traces of this activity.

Comparison of specific activity of this enzyme in virulent IP:106, normal and cholesterol passaged NIH:200 showed more than two fold higher activity in IP:106 in comparison to NIH:200 and increases in NIH:200 strain when passaged in cholesterol enriched medium (Table 22).

Action of inhibitors/activators

Data on effect of various activators and inhibitors on proteinase activity in three cultures are presented in Table 23. This activity was highly sensitive to -SH blocking agents viz. p-chloromercuribenzoate (pCMB), N-ethylmalimide (NEM) and iodoacetate and amongst these three -SH blocking agents pCMB was most potent. β-mercaptoethanol and cysteine acted as stimulating agents. Zn^{2+} and Cu^{2+} ions were inhibitory while Mg^{2+} was stimulatory. The results show that there is no variation in these effects in different strains.

The inhibition of cysteine proteinase by pCMB and NEM was completely abrogated by the prior addition of reduced glutathione, L-cysteine and β-mercaptoethanol but oxidized glutathione did not (Table 24), demonstrating the need for a reduced thiol group for this activity. Further, these compounds do not abolished the inhibitory effect of iodoacetate (another -SH group blocker), indicating the strong and irreversible effect of this compound.

Table 18: Acid phosphatase activity in different **E.histolytica** cultures.

Cultures	Specific activity (units mg protein^{-1})
NIH:200	1.96±0.25
NIH:200 (Cholesterol passaged)	3.45±0.32
IP:106	3.86±0.44

Values are mean±S.D. of three independent experiments.

Table 19: Cellular and extracellular acid phosphatase activity at different post-inoculation intervals.

Time (hr)	Cell density (million amoebae per ml)	Cells (Enzyme units)		Cells free medium (Enzyme units)	
		Without Triton X-100	With 0.1% Triton X-100	Without Triton X-100	With 0.1% Triton X-100
0	0.5	0.580	0.588	-	-
24	1.2	1.190	1.196	6.80	6.89
48	1.6	1.689	1.694	9.10	9.12
72	2.1	2.090	2.099	16.00	16.08

All the data presented are average values of 3 experiments and replicates of 3 were run for enzyme assay.

Trophozoites from 72 hr old cultures (NIH:200) were harvested by chilling and centrifugation and were washed twice to get rid of the original medium. They were used to inoculate a series of fresh culture tubes. Acid phosphatase activity was followed in the homogenized harvested cells and in the corresponding cell free medium. The cell density were determined by haemocytometer counting. For triton treatment the homogenates were preincubated with 0.1% triton X-100 at 37°C for 30 min prior to enzyme assay.

Table 20: Distribution of acid phosphatase in the peaks obtained by DEAE-cellulose chromatography from 0-60% $(NH_4)_2SO_4$ saturation fraction of **E.histolytica** (NIH:200) culture medium.

Fractions	Specific activity (Enzyme units/ mg protein)	Fold purification	PAGE profile (Relative moities)
0-60% $(NH_4)_2SO_4$ Saturation precipitate	6.34	-	-
Peak I (AP_1)	22.24	3.52	1 (0.46)
Peak II (AP_2)	49.64	7.83	2 (0.46,0.61)
Peak III(AP_3)	46.32	7.32	3(0.46,0.64, 0.66)

Table 21: Effect on acid phosphatase activity (**E.histolytica**) of activators/inhibitors/Con A/antiamoebic drugs on the different peak (AP_1, AP_2, AP_3).

Activators, inhibitors, Con A, Antiamoebic drugs	Concentration (M)	% Variation from control		
		Peak I (AP_1)	Peak II (AP_2)	Peak III (AP_3)
Sodium tartarate	10^{-3}	-3	+3	-2
Sodium fluoride	10^{-3}	-40	-41	-36
Cysteine HCl	10^{-3}	-31	-4	-20
Cysteine HCl	10^{-2}	-66	-100	-72
EDTA	10^{-3}	-54	-57	-41
Cupric chloride	10^{-3}	-11	-5	-24
Ammonium molybdate	10^{-2}	-93	-96	-69
Ammonium molybdate	10^{-3}	-20	-34	-39
Ammonium molybdate	10^{-4}	-11	-23	-29
Con A*	0.4×10^{-6}	-21	+63	+52
Con A*	0.4×10^{-5}	-38	+102	+108
Con A* + Glucose	0.4×10^{-5} 2 mg/ml	-11	-7	-26
Emetine HCl	10^{-4}	-23	-43	-39
Diloxanide furoate	10^{-4}	-29	-31	-33
Chloroquine phosphate	10^{-4}	-64	-9	-11
Chloroquine phosphate	10^{-3}	-71	-94	-52
Metronidazole	10^{-4}	-23	-58	-35

*Molecular wt. of Con A taken as 25,000, asuming it to be present as a tetramer (Reeke et al., 1975).
Values are average of three independent experimentns.

Table 22: Sulfhydryl (cysteine) proteinase activity of cell homogenate, fractions of different **E.histolytica** cultures.

Enzyme source	Specific activity*					
	NIH:200		NIH:200 (Cholesterol passaged)		IP:106	
	Without triton X-100	With triton X-100	Without triton X-100	With triton X-100	Without triton X-100	With triton X-100
Homogenate	0.514±0.018	0.820±0.037	0.979±0.044	1.663±0.073	1.229±0.058	2.364±0.051
Pellet (10,000xg,20 min)	1.410±0.063	2.766±0.131	2.544±0.113	4.690±0.123	3.015±0.150	6.196±0.177
Supernatant (10,000xg,20 min)	0.088±0.016	0.098±0.021	0.120±0.012	0.137±0.012	0.129±0.088	0.138±0.002

Values are mean±SD based on data obtained in three independent experiments.

*Change in absorbance of 0.10 hr^{-1} $(mg\ protein)^{-1}$.

Table 23: Effect of inhibitors/activators on sulfhydryl (cysteine) proteinase activity of **E.histolytica** cultures.

Inhibitors/ activators	Concentration (mM)	Relative activity		
		NIH:200	NIH:200 (Cholesterol passaged)	IP:106
None	-	100.0	100.0	100.0
EDTA	1.0	113.4	124.2	142.6
	2.0	111.3	124.8	141.3
EGTA	1.0	98.9	93.1	103.7
	2.0	116.4	110.5	115.6
N-Ethyl maleimide	1.0	61.8	53.4	59.2
	2.0	53.6	42.2	39.8
p-chloromercuri-benzoate	0.4	7.2	11.1	18.0
	0.8	2.0	9.9	4.7
Iodoacetate	1.0	14.4	21.1	17.3
	2.0	14.4	18.0	13.7
Imidazole	1.0	100.0	93.4	94.0
	2.0	96.2	91.5	93.6
O-Phenanthroline	1.0	55.5	64.3	75.3
	2.0	54.3	63.3	61.7
β-Mercaptoethanol	4.0	219.7	131.9	128.9
	8.0	219.7	165.6	143.6
Cysteine	2.5	188.8	123.4	130.2
	5.0	176.5	156.6	163.6
Zinc sulphate (Zn^{2+})	2.5	72.8	61.5	59.5
	5.0	54.3	42.2	43.8
Deoxycholate	1.0	111.1	99.0	98.7
	2.0	102.4	98.1	93.7
Magnesium sulphate (mg^{2+})	2.5	108.6	102.3	109.5
	5.0	116.0	114.6	119.6
Zinc acetate (Zn^{2+})	2.5	61.9	42.2	78.4
	5.0	77.7	21.5	31.9
Copper sulphate (Cu^{2+})	2.5	19.7	23.9	32.3
	5.0	6.1	8.4	12.7

Values are average of two independent experiments.

Table 24: Sensitivity of **E.histolytica** (NIH:200) sulfhydryl (cysteine) proteinase to thiol blocking agents and its reversal by cysteine, glutathione (red.) and β-mercaptoethanol.

Additions	Relative activity
Control (no addition)	100.00
p-Chloromercuribenzoate (0.1 mM)	20.5
+ Glutathione reduced (1.0 mM)	108.6
+ Glutathione oxidised (1.0 mM)	30.4
+ Cysteine (2.5 mM)	144.2
+ β-mercaptoethanol (4.0 mM)	137.6
N-ethyl maleimide (1.0 mM)	63.6
+ Glutathione reduced (1.0 mM)	112.3
+ Glutathione oxidised (1.0 mM)	73.4
+ Cysteine (2.5 mM)	136.2
+ β-Mercaptoethanol (4.0 mM)	150.0
Iodoacetate (1.0 mM)	15.6
+ Glutathione reduced (1.0 mM)	43.4
+ Glutathione oxidised (1.0 mM)	20.2
+ Cysteine (2.5 mM)	30.4
+ β-Mercaptoethanol (4.0 mM)	23.9

Values are average of two independent experiments.

Table 25: Effect of antiamoebic compounds on sulfhydryl (cysteine) proteinase activity of **E.histolytica** cultures.

Antiamoebic compounds	Concentration (μM)	Relative activity		
		NIH:200	NIH:200 (Cholesterol passaged)	IP:106
None	-	100.0	100.0	100.0
Emetine HCl	30	96.0	96.7	99.6
	60	92.4	89.5	93.4
Metronidazole	30	100.0	91.6	96.3
	60	100.0	89.5	92.2
Quinacrine	30	93.7	91.3	90.6
	60	81.7	80.3	86.7
Phanquinone	10	65.3	65.6	59.4
	30	20.1	15.2	10.3
Diloxanide furoate	30	100.0	101.6	96.7
	60	100.0	99.6	96.7
Iodochlorohydroxy-quinoline	30	100.0	96.5	97.3
	60	100.0	92.6	89.7
Diodoquin	30	100.0	100.0	97.2
	60	93.7	92.5	96.5

All data are average of two independent experiments.

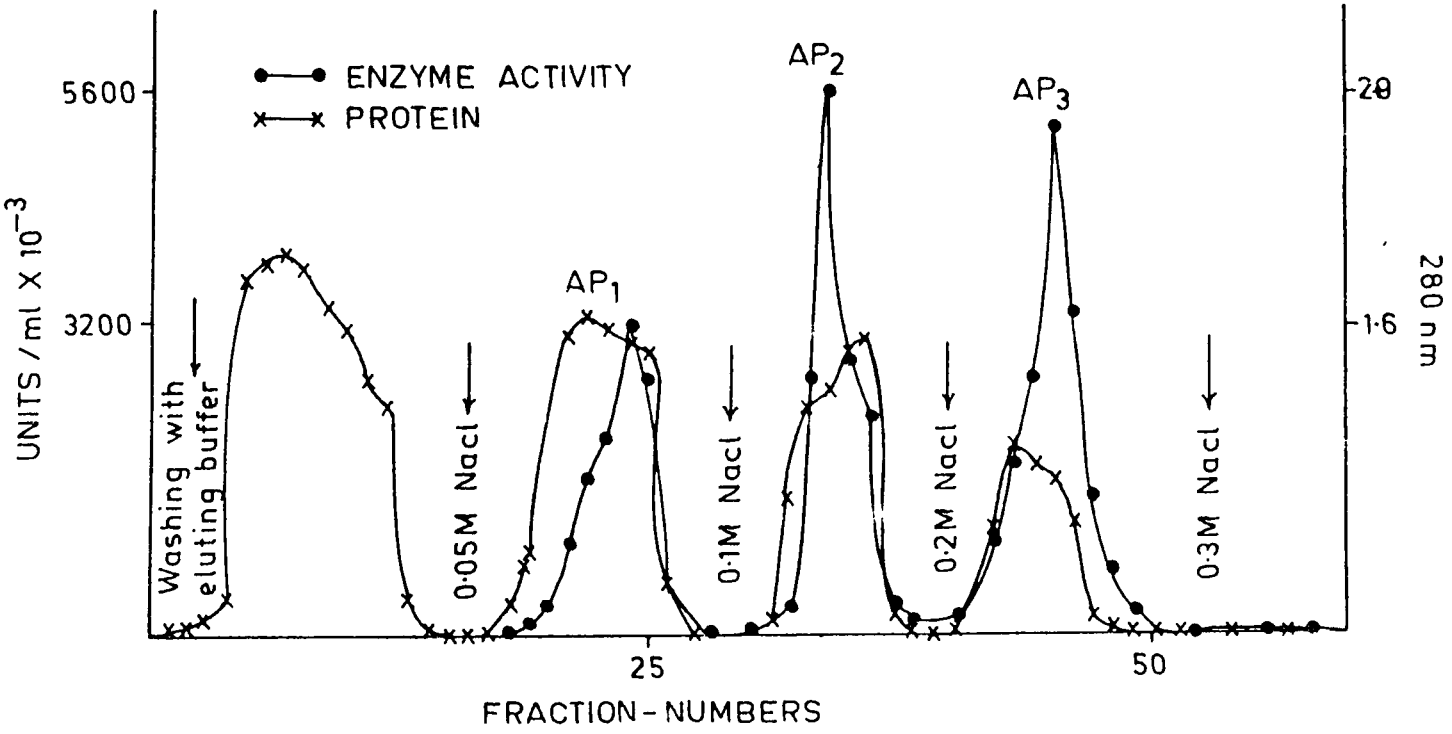


Fig.21. DEAE-cellulose column chromatography of extracellular **E.histolytica** acid phosphatase. Sample containing 150 mg protein was loaded on 2x16 cm DEAE - cellulose column, which was then serially eluted with Tris-HCl buffer (pH 7.5) and NaCl solutions of increasing concentrations (in Tris-HCl buffer) as indicated by arrows.

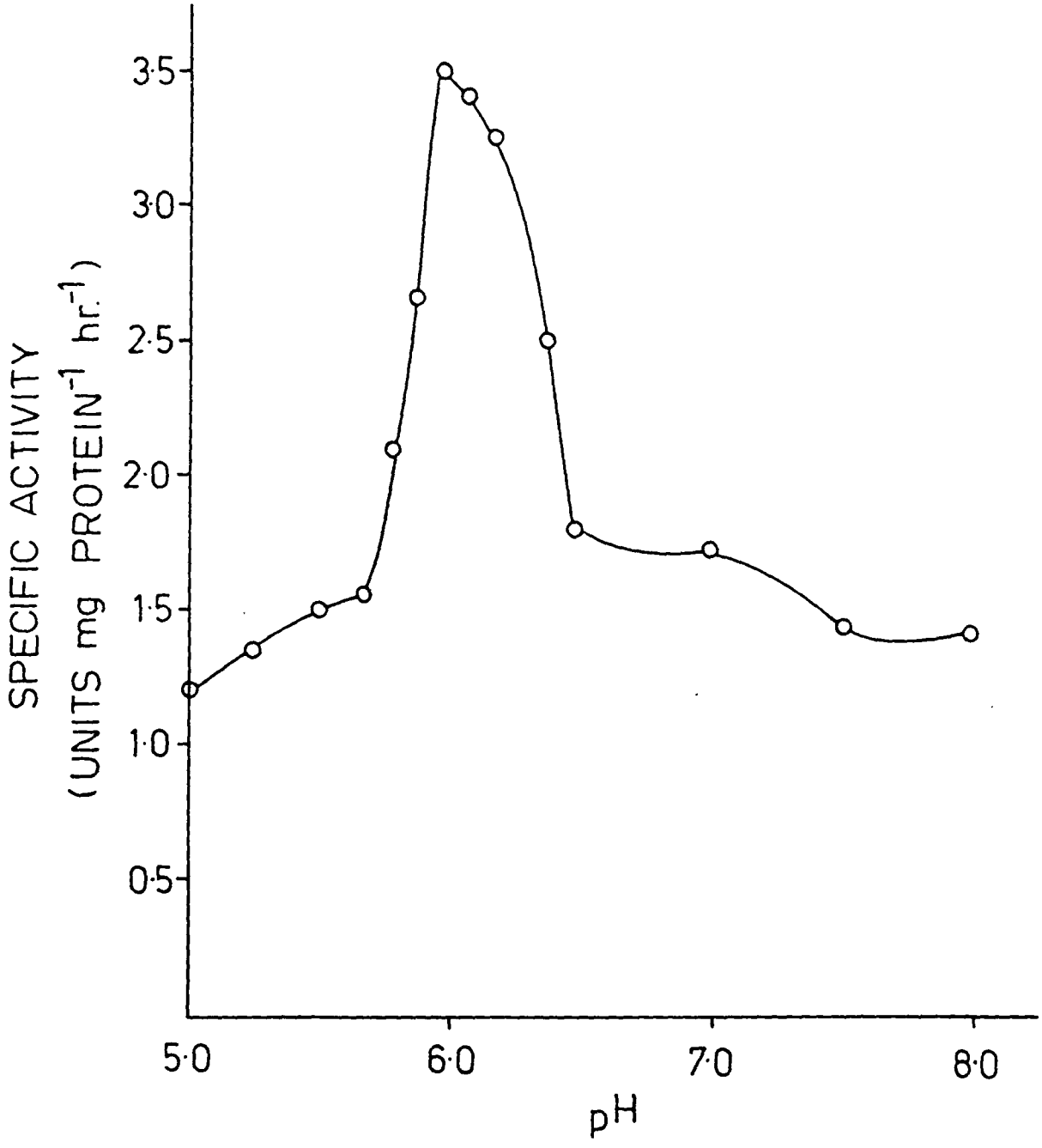


Fig.22. pH profile of sulfhydryl (cysteine) proteinase of **E.histolytica** (NIH:200).

D PYRUVATE PHOSPHATE DIKINASE : A SPECIFIC ENZYME OF E.HISTOLYTICA

Pyruvate phosphate dikinase converts phosphoenolpyruvate into pyruvate which is an important step in the glycolytic cycle of **E.histolytica**. This enzymatic reaction, in contrast to the corresponding reaction in mamalian cells, was found to require AMP and pyrophosphate instead of ADP as reported earlier by Reeves (1968). The activity was optimal at pH 6.7 (Fig. 23). Comparative results of this enzyme activity in IP:106 and NIH:200 (normal and cholesterol passaged) strains are presented in Table 26. Unlike NBT reduction, alcohol dehydrogenase and acid phosphatase activities, no significant differences were indicated in this activity in different cultures. The results on sensitivity of this enzyme to various inhibitors/activators and antiamoebic drugs are presented in Table 27, 28. The enzyme was found to be very sensitive to -SH group blocking agents pCMB and NEM but not significantly influenced by iodoacetate. Both EGTA and EDTA significantly stimulated the enzyme activity and Ca^{2+} and Zn^{2+} were inhibitory. Amongst the several known antiamoebic drugs (listed in Table 28), phanquinone (Entobex) was highly inhibitory while diodoquin caused slight inhibition. The inhibition due to phanquinone was non-competitive in nature and Ki value was 2.91 μM (Fig. 24). In contrast the activity of host pyruvate kinase (corresponding enzyme in host) of rat liver was slightly inhibited (only 10%) at 5 μM concentration of phanquinone and effect showed little further increase even at drug concentration as high as 30 μM (Fig. 25).

Table 26: Pyruvate phosphate dikinase activity of **E.histolytica** cultures.

Cultures	Specific activity (ng product formed min^{-1} mg $protein^{-1}$)
NIH:200	42.14
NIH:200 (Cholesterol passaged)	58.58
IP:106	49.87

All data are average of three independent experiments.

Table 27: Effect of inhibitors/activators on pyruvate phosphate dikinase activity in NIH:200 strain of **E.histolytica**.

Inhibitors/ activators	Concentration (mM)	Relative specific activity
Control	-	100.0
pCMB	0.02	17.0
	0.04	12.5
NEM	2.0	15.5
	4.0	14.9
Iodoacetate	2.0	100.0
	4.0	100.0
Imidazole	2.0	100.0
	4.0	94.2
O-phenanthrolene	2.0	100.0
	4.0	76.9
EDTA	2.0	125.2
	4.0	143.3
EGTA	2.0	124.0
	4.0	123.3
$CaCl_2(Ca^{2+})$	2.0	62.8
	4.0	48.6
$ZnSO_4(Zn^{2+})$	2.0	10.5
	4.0	31.1
Deoxycholate	2.0	92.2
	4.0	76.7

All data are average of two experiments.

Table 28: Effect of antiamoebic compounds on pyruvate phosphate dikinase activity in three strains of **E.histolytica**.

Antiamoebic compounds	Concentration (μM)	Relative specific activity		
		NIH:200	NIH:200 (Cholesterol passaged)	IP:106
Control	-	100.00	100.00	100.00
Emetine HCl	30	100.00	80.07	92.77
	60	88.90	78.55	89.63
Metronidazole	30	100.00	85.48	99.90
	60	98.60	81.68	96.45
Phanquinone	10	28.70	30.32	34.55
	30	19.46	14.37	20.47
Iodochlorohydroxy-quinoline	30	89.27	95.67	85.54
	60	85.50	88.16	79.72
Diloxanide furoate	30	96.50	89.79	90.65
	60	85.50	75.16	65.72
Diodoquin	30	88.26	69.37	70.39
	60	60.82	64.02	61.75

All data are average of two independent experiments.

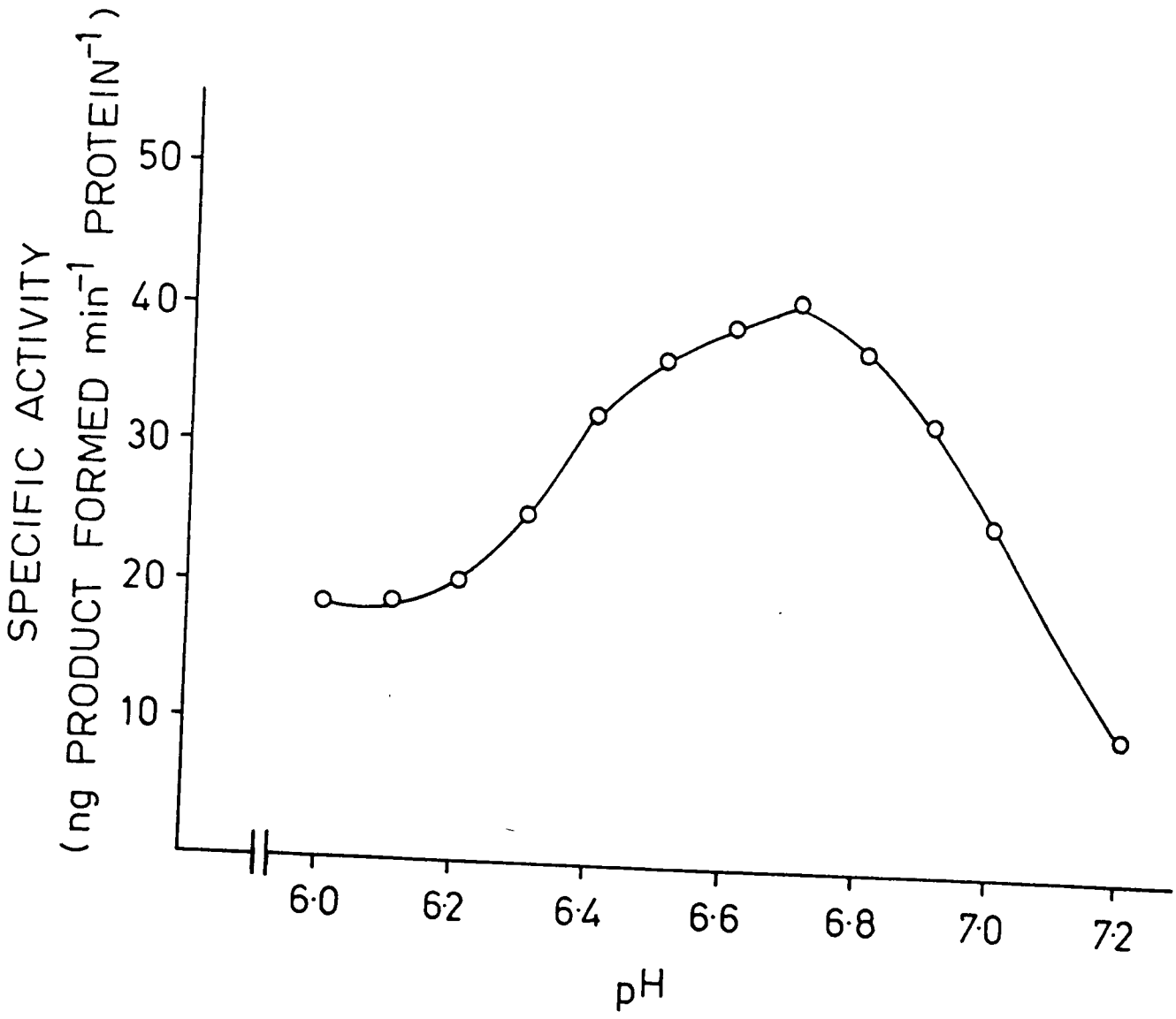


Fig.23. pH profile of pyruvate phosphate dikinase of **E.histolytica** (NIH:200).

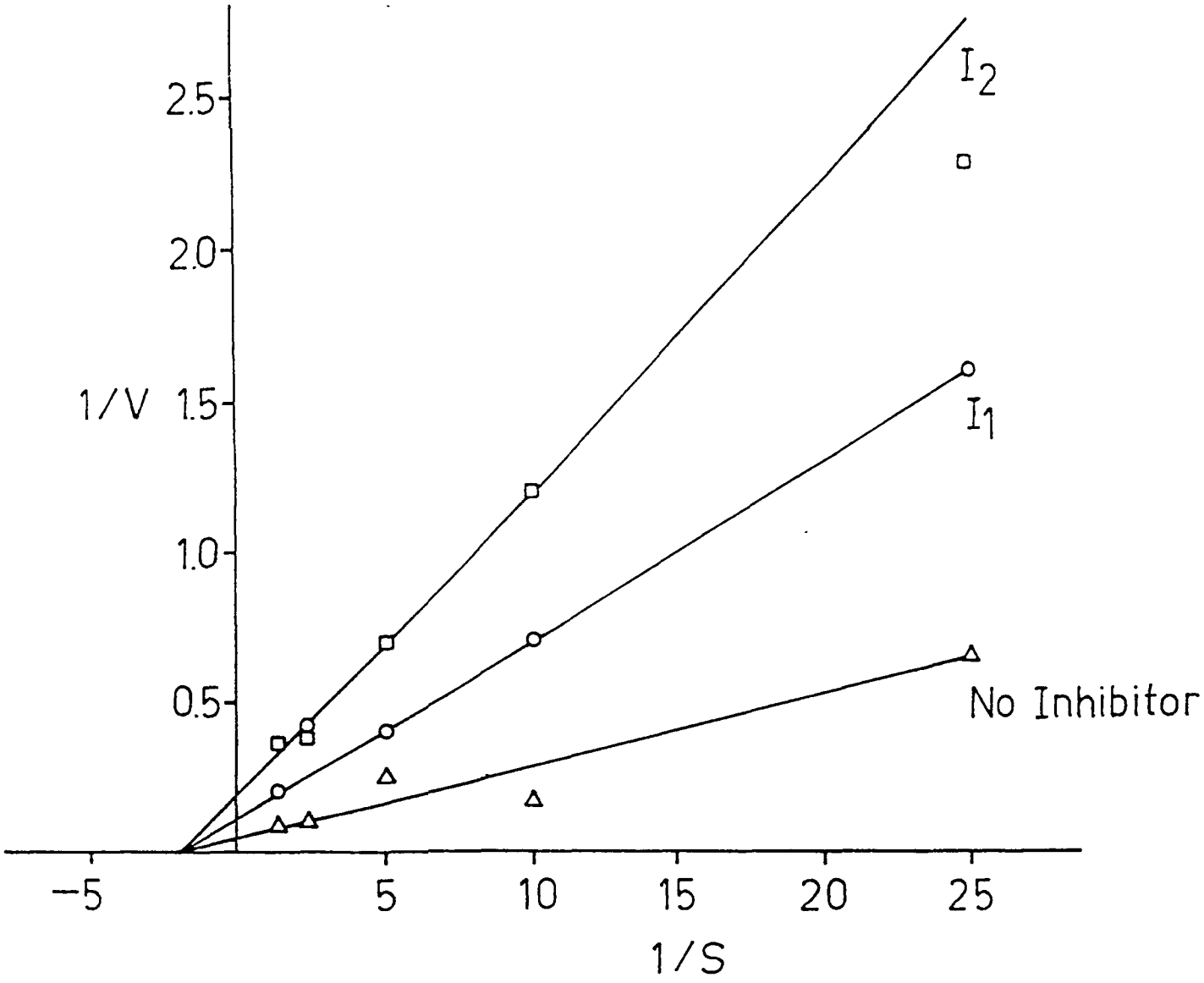


Fig.24. Non-competitive curve of pyruvate phosphate dikinase inhibition by phanquinone (Entobex, antiamoebic compound).

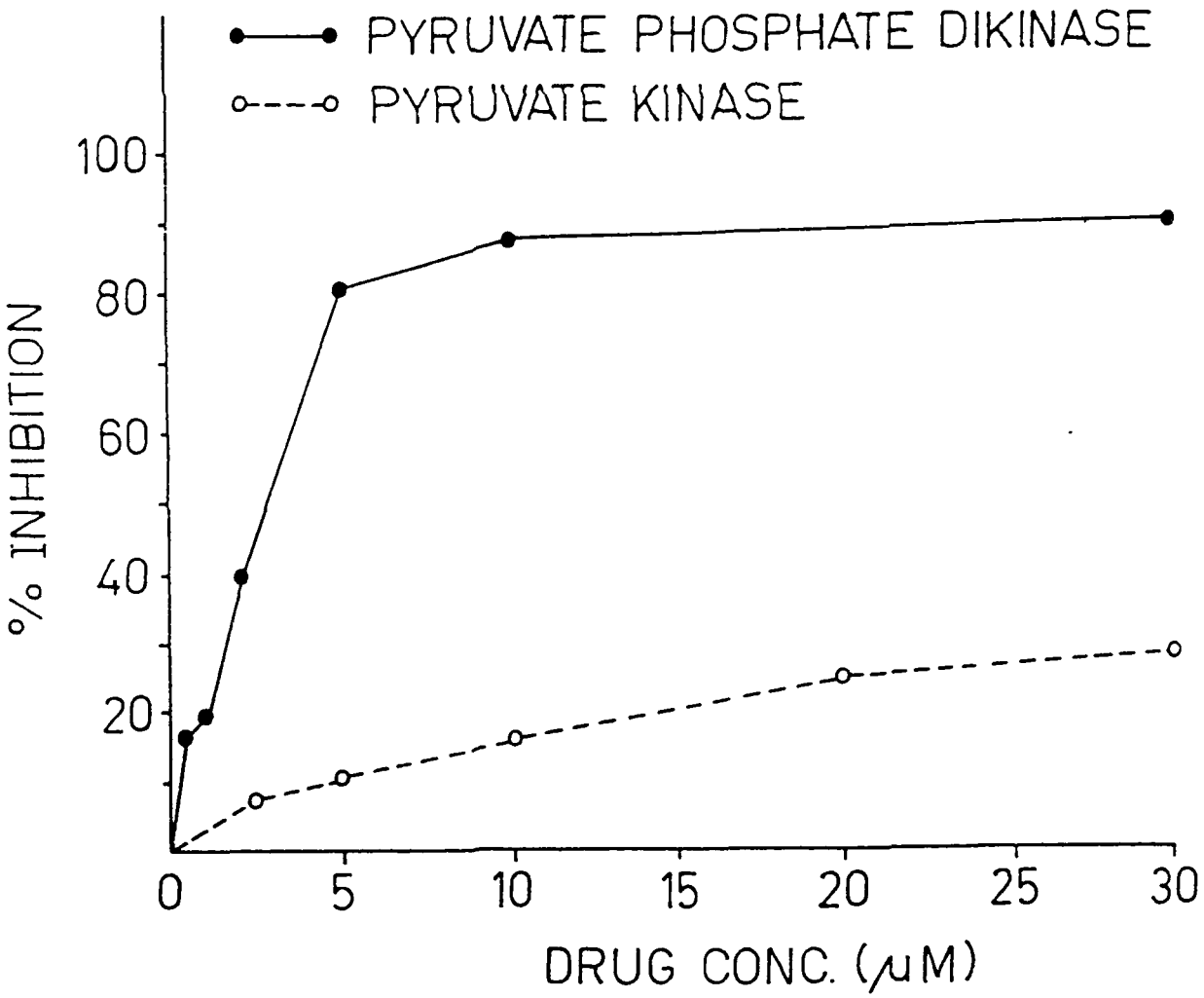


Fig.25. Inhibition curve of pyruvate kinase of rat liver (o–o) and pyruvate phosphate dikinase of **E.histolytica** (•–•) by phanquinone.

CHAPTER IV

DISCUSSION

Early efforts to correlate specific biochemical functions with **E.histolytica** virulence were hampered due to the presence of complex microflora in cultures of this amoeba. A variety of biochemical, organizational and physiological functions of **E.histolytica** have, however, been linked with its virulence with the advent of its axenic cultures (McLaughlin and Aley, 1985) and some such correlations have been discussed in the 'Introductory' section of this dissertation.

Axenization, however, often results in loss of invasive capacity of the amoeba as tested in conventional animal models, and special models have been devised to test virulence of axenised strains. Das and Ghoshal (1976) reported that capacity of the parasite to colonise and induce ulcers in rat caeca was not inherently lost in axenic NIH:200 strain and manifested itself when such cultures were supplied with bacteria together with cholesterol.

A stable marked difference was noted in the virulence of DKB and IP:106 strains as tested in new born and adult golden hamsters (Mattern and Keister, 1977; Ghadirian and Meerovitch, 1978), although both the strains were initially obtained from acute amoebiasis patients. The virulence tests performed by us in the present study by inoculating amoebic trophozoites into the liver of golden hamster, using the procedure of Dutta (1970) confirmed very low invasiveness of both DKB and NIH:200 as compared to IP:106 and the invasive capacity of NIH:200 was found to increase significantly when it was cultivated

in cholesterol enriched environment. These strains also showed marked differences in their Con A agglutinability and haemolytic potencies, two in vitro indices of **E.histolytica** virulence. These strains were, therefore, chosen for the present study; main objectives of this being elucidation of the role of certain biochemical/physiological functions of the amoeba in its invasive mechanism and to examine their potential as suitable targets for chemotherapeutic attack.

ERYTHROPHAGOCYTOSIS

A possible correlation between erythrophagocytotic capacity of **E.histolytica** and its invasiveness was indicated from very early observation of Losch (1875), who for the first time demonstrated this amoeba to be responsible for dysentery. Trissl et al. (1978) reported a correlation between virulence and erythrophagocytotic capacity in axenic amoebic cultures. Further Orozco et al. (1985) recently isolated phagocytosis deficient mutants from a highly virulent strain and observed that all these mutants were deficient in virulence as tested in animals and also in vitro cytopathic effect (Orozco et al., 1988). The present study showed much higher erythrophagocytosis in virulent IP:106 culture as compared to both NIH:200 and DKB strains using either intact erythrocytes or resealed erythrocyte ghosts. However, the clonal cultures derived from the parent DKB cultures were found to exhibit significant differences in their erythrophagocytotic capacity, supporting the possibility that amoebic population in any single culture may not be homogeneous in this respect. Thus, a strain designated as 'avirulent' on the basis of animal testing may retain some virulent

amoebic cells and the degree of invasive capacity of any culture may depend on the proportion of such heterogenous subpopulations. Further, it is noteworthy that the same DKB clone (DKB-3) which showed maximum erythrophagocytotic capacity has been found earlier to possess higher haemolytic capacity, Con A agglutinablity and greater activity of acid hydrolases (Katiyar et al., 1989).

ROLE OF SURFACE SUGARS IN ERYTHROPHAGOCYTOSIS

Ravdin and Guerrant (1982b) examined influence of various sugar molecules on adherence of **E.histolytica** with Chinese hamster ovary (CHO) cells and compared the action of these saccharides on the cytosolic and phagocytotic functions of **E.histolytica**. They observed significant inhibition of both adherence and cytolysis by N-acetyl galactosamine, although phagocytosis of these cells by the amoeba was influenced to a much lesser degree by this sugar. On this basis they suggested that different surface receptors may be involved in adherence and phagocytosis. Cano-Mancera et al. (1986) examined the effect of large number of sugars on amoebic adherence with human erythrocytes and inferred that this requires presence of at least three carbohydrate residues viz., galactose, mannose and glucose on interacting cell surface. However, they observed that different strains differ from each other in their adhesion-inhibition pattern by the carbohydrates. Contrary to inference of Ravdin and Guerrant (1982b) (in respect to CHO cell phagocytosis), present results suggest that galactose-containing moities on amoebic or erythrocyte cell surfaces are involved in the erythrophagocytotic mechanism either directly

or indirectly through controlling adhesion. Further, a similar pattern of results on erythrophagocytosis of intact RBC and its resealed ghosts indicates that cytosolic factors of these cells do not have any role in the inhibition of this process by the sugars, supporting the above inference.

Like erythrophagocytosis, internalization of labelled Concanavalin A also was found to be significantly higher in IP:106 as compared to NIH:200 and this was enhanced in the latter culture on its cultivation in cholesterol enriched medium.

OXIDATION REDUCTION FUNCTIONS

Bracha and Mirelman (1984) proposed that 'reducing power' of **E.histolytica** may have an important role in its invasion of baby hamster kidney (BHK) cells. Aust-Kettis et al. (1982), however, observed that phagocytosis of heat killed bacteria significantly enhanced the nitro-blue tetrazolium (NBT) reducing ability of **E.histolytica** and attributed this to a 'respiratory burst' type of phenomenon which is known to play an important role in the invasive process of mammalian phagocytes. The possibility that a similar phenomenon occurs in amoeba was indicated also from the present results showing marked increase in NBT reduction, as well as $^{14}CO_2$ formation from ^{14}C-glucose when erythrocytes were supplied to amoebe and by stimulation of oxygen uptake by the latter in presence of bacteria. The mechanism of reduction of this dye by **E.histolytica** was, therefore, investigated in some detail.

The results showed that this dye could be reduced by **E.histolytica** even in complete absence of any exogenous nutrients. Further, a sizable proportion of such dye reduction was brought about through organised activity of low speed sediments obtained from the homogenates. It can thus be inferred that plasma/internal membranes of the amoeba contain presumably the entire system of necessary enzymes, co-enzymes and reducible substrates, although reduction of NBT also by cytosolic constituents is not ruled out. A substrate-independent respiratory activity has recently been reported in 'mitochondria like particles' of a filarial species Setaria digitata (Raj et al., 1988) which shares several common features with oxygen utilization process in **E.histolytica** viz., cytochrome non-involvement, cyanide and rotenone insensitivity and capacity to generate reactive oxygen intermediates (ROIs), particularly H_2O_2 (Weinbach and Diamond, 1974).

The above substrate-independent NBT reducing activity of the amoeba was highly sensitive to -SH blocking agents and was inhibited also by H_2O_2. About 35% of this activity was apparently mediated through superoxide radicals as indicated from the effect of superoxide dismutase and anaerobic environment on NBT reduction. Further, since the flow of electrons from unidentified endogenous substrates to dye is subject to marked stimulation by NADH/NADPH and this is strongly inhibited by quanacrine and phanquinone, these coenzymes are probably involved in the dye reduction mechanism.

Gillin et al. (1984) inferred presence of crucial - SH groups on **E.histolytica** surface on the basis of killing effect of impermeant

thiol blocking agents, an effect which was nullified by cysteine and bovine serum albumin. The mechanism(s) reflected in substrate independent NBT reduction by this amoeba may directly or through the mediation of reactive oxygen intermediates (including H_2O_2) damage these -SH groups. This in turn may not only adversely influence -SH proteases which are probably involved in disruption of BHK cell monolayers explaining thereby the findings of Bracha and Mirelman (1984), but also -SH requiring components of its own respiratory chain. Suitable adherable phagocytosable host or bacterial cells may supply the parasite necessary substrates and/or enzymes and allow detoxification of reactive oxygen intermediates (ROIs) at their cost. Thus, under the conditions which apparently prevail in the natural host environment, the parasite may be able to utilise potentially 'suicidal' products of its respiration as its 'weapons'.

Like antiamoeba antibodies and heat killed bacteria (Aust-Kettis et al., 1982), Con A also was found to stimulate NBT reduction in **E.histolytica** and its effect was nullified by α-methyl mannoside (hapten). Con A is known to induce respiratory burst in mammalian phagocytes (Goldstein et al., 1977) and its receptors are more abundant in virulent **E.histolytica** (Prasad, 1984). Bracha and Mirelman (1983) observed that some naturally occurring Con A type of mannose binding surface components in certain bacteria or linking the bacterial cells with Con A (when such components are absent) facilitate their adherence to and ingestion by **E.histolytica** trophozoites. Further, phorbol myristate acetate another inducer of respiratory burst in phagocytes (DeChatelet et al., 1976; Chiara et al., 1989), which apparently acts

by a different mechanism than Con A was also found to stimulate NBT reduction in **E.histolytica** substantiating thereby the evidence for induction of a respiratory burst type of phenomenon in **E.histolytica** and its role in its invasive function.

NADP(H) is the coenzyme believed to be involved in the phenomenon of 'respiratory burst' in mammalian phagocytes (Fillipo, 1986). Aust-Kettis et al. (1982) presumed that NADPH flavin oxireductase (Lo and Reeves, 1980) may be responsible for NBT reduction in **E.histolytica**. However, unlike mammalian phagocytes **E.histolytica** lacks hexose monophosphate shunt pathway (Susskind et al., 1982), which is a major source of NADPH regeneration (from NADP) in mammalian system. The reduced form of this coenzyme in the amoeba is generated, presumably, at the cost of NADH either through the mediation of oxaloacetate-malate cycle (Avron and Chayen, 1988) or through direct transfer of hydrogen from NADH to NADP by a transhydrogenase. It may be significant that latter also has been shown to reside in **E.histolytica** membranes (Harlow et al., 1976), which, as mentioned, may contain all the required components to reduce NBT.

Markedly higher NBT reducing ability (in terms of specific activity) was shown by highly virulent IP:106 as compared to NIH:200 and this activity in the latter showed considerable stimulation on being maintained in cholesterol enriched environment. A similar pattern of relative specific activities in IP:106 and normal and cholesterol passaged NIH:200 culture was observed also for both NADH and NADPH linked alcohol dehydrogenases. These enzymes are believed to play an important role in regeneration of oxidised form of these coenzymes

for continuous operation of glycolytic cycle (Lo and Reeves, 1978) and may thus directly or indirectly contribute in production and/or scavanging of ROIs.

Earlier findings reported from this laboratory have shown significant stimulation of certain other glycolytic enzymes e.g. phosphoglucomutase and hexokinase through cultivation of **E.histolytica** in cholesterol enriched medium (Katiyar et al., 1987). Higher alcohol dehydrogenase specific activities in IP:106 and cholesterol passaged NIH:200 as compared to normal culture of the latter suggests that the glycolytic cycle itself may be operating at a higher pitch in the invasive forms of the amoeba. Further, this inference is supported by the observation that IP:106 can generate larger quantities of $^{14}CO_2$ from labelled glucose as compared to NIH:200 and this activity also was similarly stimulated by cholesterol.

ACID PHOSPHATASE

Possible relevance of acid phosphatase of **E.histolytica** to its virulence has been visualised by a number of investigators (Eaton et al., 1969; 1970; Lal and Garg, 1979). Eaton et al. (1969, 1970) reported an acid phosphatase containing cup shaped depression (0.1 to 0.2 μm in diameter) located beneath the membrane bound vacuole. These surface cups which were termed 'surface active lysosomes' were often seen to possess a frond-like outgrowth protruding from central surface of the evagination. These entities were considered by various workers to represent the structural basis of the cytopathic activity of **E.histolytica** (Martinez-Palomo, 1982; McLaughlin and Aley,

1985). On the other hand, existence of lysosomes in **E.histolytica** has been questioned by Rosenbaum and Wittner (1970) in view of absence of Golgi apparatus wherefrom the lysosomes are believed to originate (Whaley et al., 1972; El-Hashmi and Pittman, 1970). Recent cytochemical procedures have indicated that enzyme may be associated with both plasma-and vacuolar-membranes of the amoeba (Rosenbaum and Wittner, 1970; Serrano et al., 1977). Aley et al. (1980) demonstrated the presence of acid phosphatase activity in isolated plasma membranes of **E.histolytica**.

Earlier investigations in this laboratory (Katiyar et al., 1989) demonstrated that virulent IP:106 strain possesses higher acid-phosphatase activity as compared to less virulent strains of the amoeba and a marked quantitative increase in this occurred on repeated subculturing of the latter in cholesterol enriched environment. This observation found confirmation in the present study.

Chernov et al. (1984) recently proposed that acid phosphatase may not only be involved in utilization of endocytosed cells but also in lysing host epithelial cells at distant sites. Further, numerous functions of extra-cellular acid phosphatase at sites distal to the parasite have been envisaged in the case of Leishmania donovani (Gottlieb and Dwyer, 1982; Lovelace et al., 1986). The present investigation demonstrated progressive increase with time both in cell number and total enzyme content of harvested amoebic populations (as assayed in their homogenates) as well as of the enzyme activity released in the medium. Whereas, the enzyme content per cell did not show much variation at different culture ages, total extracellular levels of the enzyme were several fold higher as compared to the total

activity associated with cell homogenates at all the post-inoculation intervals. These results suggest that the organism activiey secretes the enzyme in its extracellular milieu during growth.

The present study further demonstrated that the fractionation of the secreted extracellular acid phosphatase by DEAE-cellulose chromatography yielded three enzyme peaks (AP_1, AP_2 and AP_3). The PAGE of the enzyme in these three peaks showed 1, 2 and 3 enzyme bands respectively. However, one of the band in all these three peaks gave common Rm value of 0.46. Numerous examples have been documented in literature which show that diffuse enzyme bands separated through unidimentional PAGE may actually be heterogenous and may get resolved into multiple molecular forms of the enzyme on further analysis. It has also been proposed that such enhanced heterogenity may arise from variable post-translational modification of the core enzyme-protein through glycosylation (Barbaric et al., 1980; Schoenholzer et al., 1985), and phosphorylation (Frevert and Ballou, 1982). This, possibly provides an explanation for the present observation that acid phosphatase fractions which differ in their elutability from DEAE-cellulose share an enzyme band having the same Rm value. The enzyme in all the three peaks showed insensitivity to tartrate but was inhibited by fluoride, Cu^{+2}, EDTA, ammonium molybdate and surprisingly also by cysteine. Concanavalin A, however, caused a concentration-dependent inhibition of the enzyme in AP_1 but stimulated this activity in AP_2 and AP_3 at $0.4x10^{-6}$ and $0.4x10^{-5}$M concentrations, respectively. Both stimulatory and inhibitory responses showed reversal when glucose was simultaneously added with lectin. These

3 peaks showed presence of carbohydrate as tested by the phenol sulfuric acid method (Montgomery, 1957). The above results suggest heterogeneity of secreted acid phosphatase of **E.histolytica** and presence of oligosaccharide moities in these enzyme molecules which possibly may have some role in regulation of their activity.

SULFHYDRYL PROTEINASE (CYSTEINE PROTEINASE)

Possible involvement of proteinase activity of **E.histolytica** in its host-tissue lytic functions has been suspected almost since the identification of its pathogenic nature. However, early investigations using complex xenic cultures failed to demonstrate any reliable correlation between this enzyme activity and amoebic virulence (Neal, 1960; Jarumilinta and Maegraith, 1969).

In vitro action of **E.histolytica** cells and their homogenates on mammalian monolayer cultures by several groups of investigators (Ravdin et al., 1980; Bos, 1979; Lushbaugh et al., 1979; Bos et al., 1980; Mattern et al., 1980; McGowan et al., 1982) indicated that amoeba can destroy such cultures through contact cytolysis as well as through a soluble toxin acting at some distance from the amoebae. Such effect of the amoeba was inhibited by proteinase inhibitors like α-globulins (Lushbaugh et al., 1981), leupeptin and antipain (McGowan et al., 1982; Lushbaugh et al., 1984b, North et al., 1990), suggesting that proteolytic enzymes may play a part.

McLaughlin and Faubert (1977) for the first time partially purified an acid proteinase and a neutral proteinase from an axenic culture. The former exhibited maximum activity at pH 3.5 on haemo-

globin as substrate and the latter at pH 6.0 with azocasein. Two enzymes on the basis of their sensitivity to various inhibitors and activators and other properties seemed to resemble cathepsin D and cathepsin B respectively.

Bos (1979) isolated and characterised an intracellular toxin from axenic HK-9 culture of **E.histolytica** which had a cytopathogenic effect on monolayers of baby hamster kidney cells. Subsequent work of Bos et al. (1980) indicated that this cytotoxicity of trophozoite extracts was activated in vitro by thiol groups and was inhibited by iodoacetate and also a high molecular weight fraction of non-immune serum. They suggested that contact dependent lysis and toxin induced cytopathic effect may both be caused by the same substance and pointed out its similarity with neutral sulfhydryl proteinase of McLaughlin and Faubert (1977).

Lushbaugh et al. (1985) undertook intensive investigations to characterise the factors responsible for this cytotoxic activity of **E.histolytica** and showed that amoebal cytotoxic neutral porteinase activity had an isoelectric point and molecular weight similar to that of mammalian cathepsin B and found a correlation between this activity and strain virulence.

The present study examined proteinase activity of IP:106 and of normal and cholesterol passaged NIH:200 cultures using azocasein as the substrate and assay conditions generally similar to those used by McLaughlin and Faubert (1977). The activity was optimum at pH 6.0 and was strongly inhibited by all the -SH group blocking agents. Further, the inhibition due to pCMB and NEM was reversed

by reduced -SH donors viz., glutathione (red.), cysteine and β-mercaptoethanol. Further, bulk of this activity (approximately 95%) was found to reside in the sedimentable components and showed substantial stimulation by triton X-100 in all the three cultures. This indicates that this is probably located in plasma and/or intracellular membranes of the cell and displays latency, as reported also for several 'lysosomal' enzymes of this amoeba (Van Vleiet et al., 1971; Trissl, 1983; McLaughlin and Meerovitch, 1975b). A comparison of relative specific activities in the three cultures indicated its correlation with amoebic virulence supporting thereby the mentioned findings of Lushbaugh et al.(1985).

GENERAL SENSITIVITY OF E.HISTOLYTICA FUNCTIONS TO -SH GROUP BLOCKERS AND PHANQUINONE (4, 7-PHENANTHROLINE QUINONE)

In addition to NBT reducing and neutral proteinase activities, alcohol dehydrogenase and pyruvate phosphate dikinase also showed sensitivity to -SH blocking agents. These findings suggest that functional -SH groups may be involved possibly in a large number of biochemical functions of **E.histolytica**. This seems particularly interesting in light of an obligatory requirement of cysteine by this amoeba in its axenic growth media. This aminoacid, moreover, has been found to be vital for the survival of the amoeba even under anaerobic conditions (Band and Cirrito, 1979; Gillin and Diamond, 1981) indicating that this probably plays some direct role in growth and survival of the amoeba besides maintaining low redox potential.

Interestingly all the four biochemical activities of **E.histolytica** named in preceding paragraph showed considerable general inhibition also with the antiamoebic drug phanquinone. This drug, as mentioned, exhibited the property of spontaneously oxidising both NADH and NADPH. It may be interesting to investigate whether the reduced form of these nucleotides are in some way involved in maintaining vital -SH groups of functional biochemical entities in reduced state.

PYRUVATE PHOSPHATE DIKINASE

As mentioned under 'Introduction' pyruvate phosphate dikinase (PPD) of **E.histolytica** which is functionally analogous to pyruvate kinase of mammalian cells is characterised by its requirement of PPi and AMP (Reeves, 1968). Besides, this enzyme probably plays an important role in regulation of cellular pyrophosphate level and prevent its inhibitory effect on macromolecular synthesis (Wood et al., 1977). In view of these unique features, the enzyme may form a vulnerable target for selective chemotherapy against the parasite. Among the several antiamoebic drugs tested, phanquinone showed a very high degree of sensitivity towards this enzymes of **E.histolytica**. Thus, as low as 2.91 μM drug caused 50% inhibition of PPD (Ki:2.91 μM), while several fold higher phanquinone concentration was required to induce the same degree of effect in case of other drug-sensitive parameters viz., NBT reduction and cysteine proteinase. This inhibition of amoebic PPD by the drug was non-competitive as indicated from Lineweaver burk analysis and appeared to be selective in so far as the pyruvate kinase of rat liver showed little sensitivity under comparable conditions.

ACTION MECHANISM OF CHOLESTEROL

Stimulation of a variety of biochemical functions of low virulence cultures of **E.histolytica** has been observed on their cultivation in cholesterol enriched environment. It has thus been reported that cultivation of NIH:200 strain of **E. histolytica** in presence of high levels of this sterol caused marked increase in the specific activities of several lysosomal enzymes viz., acid phosphatase (Pandey et al., 1977; Katiyar et al., 1988), acid hydrolases (Lal and Garg, 1979). Further, this enhanced greatly its Con A agglutinability, haemolytic potency and specific activities of phosphoglucomutase and hexokinase (Katiyar et al., 1987). The present investigation further extended this list and demonstrated that cultivation in presence of high levels of this sterol in the medium markedly stimulated erythrophagocytotic and CO_2 producing abilities of NIH:200 strain. This also considerably increased NBT reducing-alcohol dehydrogenase and cysteine proteinase specific activities. It is difficult to conceive any unified direct mechanism whereby cholesterol may influencing such a large array of biochemical functions which incidently all show higher levels in the more virulent IP:106 strain. These findings thus support the concept putforth by Martinez-Palomo (1982) that cholesterol enriched environment may facilitate selection of pre-existing virulent subpopulations in **E.histolytica** cultures. This hypothesis found support also from recent observations of Katiyar et al. (1989) that several virulence-related biochemical activities exhibited significant quantitative differences in the clonal cultures derived from the same 'avirulent' parent strain and from the observed immunity of the clones towards choles-

terol. The low phagocytotic ability of the clone which has lower activities of other parameters, examined by Katiyar et al. (1989), indicates that invasive property of the amoeba may depend on a large number of factors which are simultaneously influenced by cholesterol. Such linkage of virulence-related functions is indicated also from the observations reviewed by Gitler and Mirelman (1986), indicating parallel increase of protease and collagenase, contact mediated killing of target cells and resistance to direct and indirect complement cascades following a variety of treatment (including exposure to cholesterol), which enhance **E.histolytica** virulence. Such bunched stimulation of multitude of factors by cholesterol may facilitate parasitic invasion and also its survival in the host system through blunting latters defences and more efficient operation of its own energy generating mechanisms.

CHAPTER V

SUMMARY AND CONCLUSIONS

Entamoeba histolytica attained considerable medical interest with the discovery of Losch, a Russian physicist, in 1875, that this amoeba was the cause of dysentery in man. However, many infected individuals showing presence of cystic and tropic forms of the amoeba in their stools, were often found to be free from any symptoms of this disease. Variable invasive potency of different **E.histolytica** isolates has also been seen in experimental animals and quite often such cultures, which were initially pathogenic, lose their virulence on prolonged cultivation particularly under axenic conditions as tested in conventional animal models. Such attenuated cultures may, however, generate pathological manifestations in specially devised models and generally regain invasiveness by repeated cultivation in cholesterol enriched culture media.

The main interest of the present study was to investigate biochemical differences in 'invasive' and 'non-invasive' **E.histolytica** strains and modification of such parameters by cultivation of amoeba in cholesterol enriched medium. Further, salient properties of these biochemical/physiological functions including their sensitivity to specific activators/inhibitors and antiamoebic drugs were also investigated.

STRAIN VIRULENCE

The **E.histolytica** cultures used in the study viz., DKB, NIH:200 and IP:106 were all obtained originally from acute amoebiasis patients

but DKB and NIH:200 showed a much lower degree of virulence compared to IP:106 as tested in terms of lesion forming ability in the liver of hamsters. Further, the capacity of NIH:200 strain to infect animals (hamsters) was found to increase considerably after its subculturing in cholesterol enriched medium. The same order of virulence was indicated from tests of Concanavalin A (Con A) agglutinability of the amoebic cells and haemolytic activity of their homogenates, which are in vitro indices of **E.histolytica** virulence.

ERYTHROPHAGOCYTOSIS

The virulent IP:106 culture showed significantly higher erythrophagocytotic capacity as compared to DKB and NIH:200 strains. Statistically significant differences were, however, indicated in the clonal cultures derived from the DKB strain which exhibited lowest erythrophagocytotic capacity amongst the three strains. The same DKB clone (DKB-3) which showed maximum erythrophagocytotic capacity was found earlier in our laboratory to possess highest haemolytic capacity, Con A agglutinability and acid hydrolase activities amongst the tested clones. Serial passage of NIH:200 culture through cholesterol-enriched medium resulted in considerable increase in the erythrophagocytotic capacity of the culture. The same relative order of phagocytosis was indicated in the three cultures (DKB, NIH:200 and IP:106) when ^{14}C-sucrose-loaded RBC ghosts were supplied to amoebic cells and uptake of radioactivity was measured.

A number of sugars viz., galactose, N-acetylgalactosamine, lactose and fructose inhibited erythrophagocytosis in both NIH:200

and IP:106 strains of **E.histolytica**. N-acetylgalactosamine also reduced phagocytosis of ^{14}C-sucrose loaded RBC ghosts.

The rate of $^{14}CO_2$production from ^{14}C-U-glucose was considerably higher in IP:106 as compared to NIH:200 and increased when the latter was cultivated in cholesterol enriched medium. Addition of RBC stimulated this activity of **E.histolytica** in all the three cultures (IP:106, normal and cholesterol passaged NIH:200); maximum increase being shown by IP:106. Further, a similar pattern of relative ^{125}I-labelled Con A internalization was noted in these cultures which showed time dependence and marked reduction in presence of α-methyl mannoside (specific hapten of Con A), while bovine serum albumin on the other hand elicited stimulation.

OXIDO-REDUCTIVE FUNCTIONS

E.histolytica can reduce nitroblue tetrazolium (NBT) in Hank's balanced salt solution to almost the same extent as in Eagle's medium. Further, this was stimulated only to a minor degree by substrates viz., glucose, pyruvate and DL-serine, known to support its respiratory activity (O_2 uptake). However, both NADH and NADPH increased NBT reduction several fold, the effect being greater with latter. A sizable proportion to this endogenous dye-reducing capability was associated with low speed sediments obtainable from amoebic homogenates which shared also the bulk of ^{125}I label (when the homogenate were prepared after surface-labelling of the cells with Na ^{125}I). Conversion of the dye to formazan was strongly inhibited by -SH blocking agents but was not influenced by rotenone and antimycin A. The activity

was inhibited also by H_2O_2 while catalase stimulated this. Superoxide dismutase only slightly curtailed NBT reduction in intact cells, but inhibited this in homogenates in a concentration dependent manner to a maximal extent of 35%. Almost the same degree of curtailment in this activity was induced by anaerobic conditions. Both phorbol myristate acetate and concanavalin A stimulated the activity in intact cells; the effect of the latter being nullifiable by α-methyl mannoside.

This substrate-independent reduction of NBT was strongly inhibited by antiamoebic drugs, phanquinone and quinacrine, and also by metal chelator, o-phenanthroline.

Mixture of amoebic and red blood cells reduced NBT to a much higher extent than total reduction accounted in terms of the activities observed with these two types of cells separately indicating thereby that erythrophagocytosis stimulates reduction of NBT in amoeba. Further, addition of Escherichia coli to the **E.histolytica** cell suspension also increased the rate of O_2 uptake by this amoeba.

Comparison of specific activities of NBT reduction and alcohol dehydrogenase in the virulent IP:106, the normal and cholesterol passaged NIH:200 showed more than two fold higher values of both these parameters in IP:106 and cholesterol passaged NIH:200 as compared to normal (unpassaged) NIH:200. The alcohol dehydrogenase activity highly favoured production of alcohol from acetaldehyde and the backward reaction showed a very poor rate. Further, the enzyme showed a much higher activity in presence of NADH as compared to NADPH. This activity of **E.histolytica** was highly sensitive to -SH blocking agents.

It has been postulated, on the basis of above results, reactive oxygen metabolites generated by **E.histolytica** are potentially self-injurious. However, the parasite can readily detoxify them at the cost of suitable adherable/phagocytosable host or bacterial cells and can thus use them as 'weapons' in the host system.

ENZYMES RELATED TO E.HISTOLYTICA VIRULENCE

Greater acid phosphatase activity in the homogenates of IP:106 as compared to NIH:200 and its stimulation in the latter through cultivation in cholesterol enriched medium was reported earlier from our laboratory. This finding was confirmed in the present investigation.

A comparison of total acid phosphatase content of **E.histolytica** cells (as assayed in its homogenates) versus the enzyme activity released in growth medium at different post-inoculation intervals indicated progressive increase with time both in cell number and total enzyme content of harvested amoebic populations as well as enzyme activity released in the medium. The enzyme content per cell did not show much variation at different culture ages, however, total extracellular levels of the enzyme were several fold higher as compared to the activity of the cellular populations at all the post-inoculation intervals suggesting that the enzyme is actively secreted by these cells in the growth medium. Triton X-100 did not influence the enzyme activity in either cellular homogenates or cell free medium.

Fractionation of the precipitate obtained from the cell-free medium at 60% ammonium sulfate saturation by DEAE-cellulose chromatography yielded 3 distinct peaks (AP_1, AP_2 and AP_3) of the enzyme

activity on stepwise elution with 0.05, 0.1 and 0.2 M sodium chloride. The PAGE of the enzyme in AP_1 gave 1 enzyme band (Rm: 0.46), 2 bands in AP_2 (Rm : 0.46 and 0.61) and 3 bands in AP_3 (Rm: 0.46, 0.64, 0.66).

The activities of three peaks showed resistance to tartarate, but was inhibited by fluoride, cupric chloride, ethylenediamine tetraacetic acid, ammonium molybdate and cysteine. Concanavalin A however, caused a concenntration dependent inhibition of enzyme in AP_1 but stimulated this activity in AP_2 and AP_3. Glucose (2 mg/ml) reversed this stimulatory or inhibitory effect of Con A. All tested antiamoebic drugs were inhibitory towards this enzyme of **E.histolytica**.

A pattern similar to that of acid phosphatase was noted for relative specific activities of proteinase in IP:106 and normal and cholesterol passaged NIH:200 using azocasein as substrate. This was predominantly associated with the sedimentable fraction (10,000xg, 20 min) and was significantly stimulated by 0.5% triton X-100. Further, it showed optimum activity at pH 6.0 and was highly sensitive to -SH blocking agents, Zn^{+2} and Cu^{+2} while β-mercaptoethanol and cysteine acted as stimulants. Glutathione (red.), L-cysteine and β-mercapto-ethanol reversed the inhibitory action of p-chloromercuribenzoate and N-ethylmaleimide. Antiamoebic compound, phanquinone, also inhibited this activity.

PYRUVATE PHOSPHATE DIKINASE

Generation of pyruvate from phosphoenolpyruvate in **E.histolytica** (NIH:200) is carried out by pyruvate phosphate dikinase (PPD), an

enzyme which is characterized by requirement of pyrophosphate and AMP instead of ADP in case of functionally analogous mammalian pyruvate kinase. The enzyme of **E.histolytica** showed optimal activity at pH 6.7 and was highly sensitive to p-chloromercuribenzoate and N-ethylmaleimide but not iodoacetate. Both EDTA and EGTA significantly stimulated PPD activity and Ca^{+2} and Zn^{+2} were inhibitory. Antiamoebic compound phanquinone was highly inhibitory (70% inhibition at 10 μM concentration) while diodoquin also caused slight inhibition (40% at 60 μM concentration). The inhibition due to phanquinone was non-competitive in nature (Ki: 2.91 μM). In contrast, the pyruvate kinase activity of rat liver homogenate was only slightly inhibited (10%) at 5 μM phanquinone concentration and the effect showed little further increase even at a drug concentration as high as 30 μM.

The study has generally inidcated that the virulence of **E.histolytica** may not be linked to any single biochemical function but may depend on a multitude of factors which are all stimulated through cultivation of avirulent strains in cholesterol enriched medium, in a bunched manner. This lends support to the view that virulent sub-populations may in fact preexist in such cultures which get enriched through some selective process in presence of high level of cholesterol in the growth environment. The work also suggests that thiol groups may be involved in several important functions of this amoeba. This feature and specific requirement of pyrophosphate by this parasite may be useful in developing new chemotherapeutic strategies against this pathogen.

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